AD_____

Award Number: W81XWH-04-1-0286

TITLE: The Role of the Sonic Hedgehog Pathway for Prostate Cancer Progression

PRINCIPAL INVESTIGATOR: Jingwu Xie, Ph.D.

CONTRACTING ORGANIZATION: University of Texas Medical Branch
Galveston, Texas 77555-0657

REPORT DATE: February 2005

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	February 2005	Annual (30 Jan 2004 - 29 Jan 2005)

4. TITLE AND SUBTITLE
The Role of the Sonic Hedgehog Pathway for Prostate Cancer Progression

5. FUNDING NUMBERS
W81XWH-04-1-0286

6. AUTHOR(S)
Jingwu Xie, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Texas Medical Branch
Galveston, Texas 77555-0657

E-Mail: jinxie@utmb.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates: All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The hedgehog pathway plays a critical role in the development of prostate. However, the role of the hedgehog pathway in prostate cancer is not clear. Here we report that activation of the hedgehog pathway occurs frequently in advanced human prostate cancer. We find that high levels of hedgehog target genes, PTCH1 and hedgehog-interacting protein (HIP), are detected in over 70% of prostate tumors with Gleason scores 8–10, but in only 22% of tumors with Gleason scores 3–6. Furthermore, four available metastatic tumors all have high expression of PTCH1 and HIP. We find that Su(Fu) protein is undetectable in 11 of 27 PTCH1 positive tumors, two of them contain somatic loss-of-function mutations of *Su(Fu)*. Furthermore, expression of sonic hedgehog protein is detected in the majority of PTCH1 positive tumors (24 out of 27). High levels of hedgehog target genes are also detected in three prostate cancer cell lines (DU145, LN-Cap and PC3). We demonstrate that inhibition of hedgehog signaling by smoothened antagonist, cyclopamine, suppresses hedgehog signaling, down-regulates cell invasiveness and induces apoptosis. All these data suggest a significant role of the hedgehog pathway for cellular functions of prostate cancer cells. Our data indicate that activation of the hedgehog pathway, through loss of Su(Fu) or overexpression of sonic hedgehog, may involve tumor progression and metastases of prostate cancer. Thus, targeted inhibition of hedgehog signaling may have significant implications of prostate cancer therapeutics.

14. SUBJECT TERMS
prostate cancer, suppressor of fused, hedgehog, patched

15. NUMBER OF PAGES
34

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

This proposal is to evaluate the role of the hedgehog pathway in prostate cancer in clinical specimens, and to identify the molecular basis of hedgehog mediated tumor formation using cultured cell lines.

Body

Good progress has been made on this project. We have one manuscript published and another manuscript in preparation. Specifically, we have demonstrated that the hedgehog signaling pathway is frequently activated in advanced prostate cancer (Molecular Cancer 3: 29, 2004, see Appendix for details). This significant discovery led to two news releases (see Appendices 2 and 3 for details). In addition, an invited review on this research area was generated during this process.

Task 1: Examine activation of hedgehog signaling activation in prostate cancer

We have successfully collected over 200 prostate cancer specimens last year. Fifty five of them are TURP (transurethral resection of the prostate) specimens, and the rest are from prostatectomy of patients with different stages of prostate cancer. We mainly focused on these 55 TURP specimens in our studies because of the following reasons. First, prostatectomy specimens are heterogeneous, with variations within a paraffin block. In contrast, TURP specimens are relatively homogeneous in tumor morphology. Second, TURP specimens can be used for other analyses such as real-time PCR and Western blotting.

Through examination of these 55 TURP specimens using specific antibodies, we found that 27 specimens contain activated hedgehog signaling (see Table 1 below), 11 tumors with no detectable Su(Fu). In addition, we discovered that Shh expression was elevated in 28 specimens (see Table 1 for details).

Figure 1 shows immunohistostaining of PTCH1 in two tumors. One tumor had no expression of PTCH1 (Fig. 1a) whereas the other with a high level of PTCH1 (Fig. 1b). The staining was specific to the PTCH1 peptide from which the antibodies were made (Fig. 1c) because no positive signals were seen in the presence of excess of the PTCH1 peptide.

Table 1 Summary of protein expression in prostate cancer specimens

Tumor grade											
Gleason 3-6 (18) 4/18				Gleason 7 (15) 7/15				Gleason 8-10 (22) 16/22			
18 Su(Fu) Positive	4 PTCH1 Positive	4 HIP positive	10 Shh positive 10/18	13 Su(Fu) Positive	7 PTCH1 Positive	7 HIP positive	4 Shh positive 4/15	13 Su(Fu) Positive	16 PTCH1 Positive	16 HIP positive	14 Shh positive 14/22
18/18				13/15				13/22			

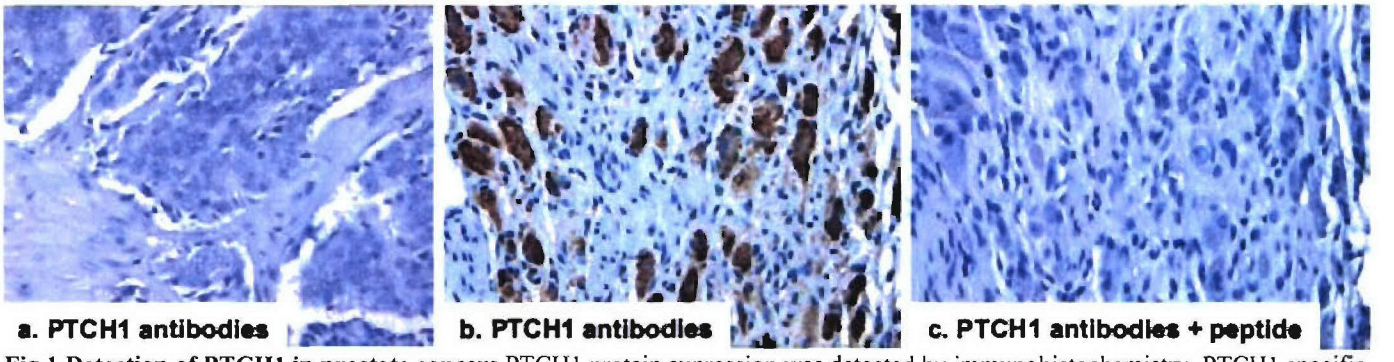


Fig.1 Detection of PTCH1 in prostate cancers PTCH1 protein expression was detected by immunohistochemistry PTCH1 specific antibodies (Santa Cruz Biotechnology Inc Cat# 6149; 1:50 dilution).

To confirm the data from PTCH1, we examined expression of another hedgehog target gene, HIP (hedgehog-interacting protein) in these prostate cancer specimens. We found that HIP was highly expressed in tumors with PTCH1 expression (see Table 1), and the pattern of HIP expression is very similar to that of PSA (prostate specific antigen), a sensitive marker for early prostate cancer. Thus, it appears that hedgehog signaling is frequently activated in prostate cancer.

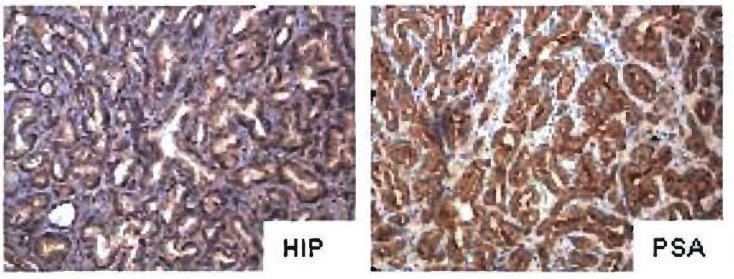


Fig. 2 Detection of HIP expression in prostate cancers Expression of HIP, another hedgehog target gene and a component of hedgehog signaling pathway, was detected using HIP specific antibodies (R&D systems, Cat# AF1568; 1:50 dilution). The pattern of HIP expression was similar to that of PSA, a serum marker for prostate cancer.

In these specimens (n=55), expression of hedgehog target genes, PTCH1 and HIP, occur only in the tumor, not in the adjacent normal prostate tissues or in the stroma (data not shown here), indicating that hedgehog signaling is mainly activated in the tumor.

Further analysis indicates that hedgehog signaling is more frequently activated in tumors with high Gleason scores (see Table 1). In tumors with Gleason scores 3-6, four out of eighteen (22.2%) tumors had activated hedgehog signaling. Seven of fifteen tumors (46.7%) with Gleason scores 7 had elevated hedgehog signaling while sixteen out of twenty two (72.7%) tumors with Gleason scores 8-10 had hedgehog signaling activation. Statistic analysis using Binomial Proportions showed that the difference between tumors with Gleason scores 3-6 and tumors with Gleason scores 8-10 are significant (p value= 0.00148 below 0.05). These data indicate hedgehog signaling activation is associated with prostate cancer progression.

Expression of Su(Fu) and sonic hedgehog in prostate cancer

Next we evaluate expression of Su(Fu) and sonic hedgehog in these prostate cancer specimens. Using antibodies specific to Su(Fu) (Santa Cruz Biotechnology Inc. Cat# 10933, 1:40 dilution), we detected Su(Fu) in all 18 prostate cancer specimens with low Gleason scores (Table 1). However, we only found Su(Fu) expression in 26 of 37 tumors with Gleason scores 7-10 (Table 1). The differences were significant (P value < 0.05, using Binomial Proportions analysis). Loss of detectable Su(Fu) was correlated with elevated expression of PTCH1 and HIP proteins, suggesting that Su(Fu) loss may be responsible for hedgehog signaling activation in these tumors.

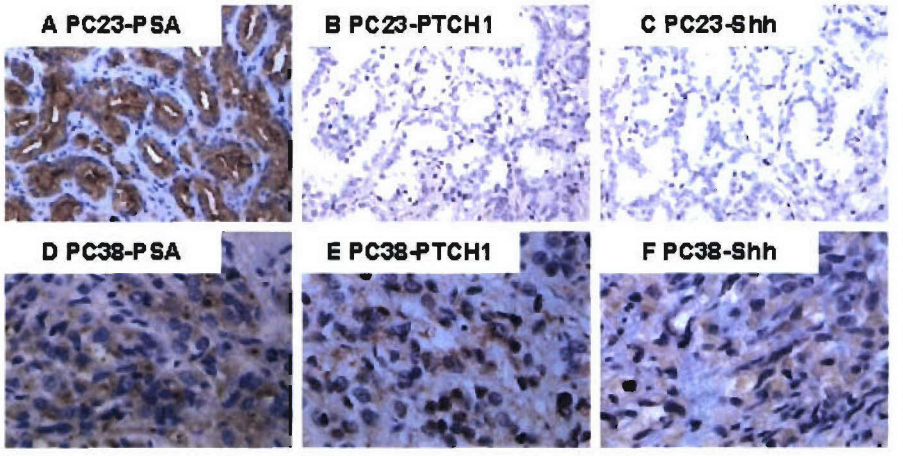


Fig. 3 Expression of PTCH1, Shh and PSA in prostate cancer Expression of PTCH1, Shh and PSA was detected by immunohistochemistry. The protein expression patterns of PTCH1 and Shh were compared with that of PSA, a marker for prostate cancer. PC23 had no detecatble expression of PTCH1 and Shh whereas PC38 has elevated expression of PTCH1, Shh and HIP. Positive signals were in brown.

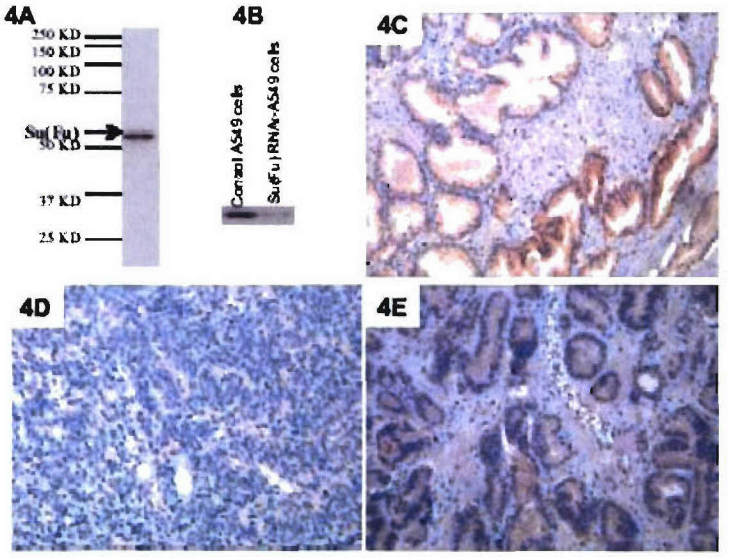


Fig. 4 Detection of Su(Fu) in prostate cancer specimens Su(Fu) antibodies (Santa Cruz Biotechnology Cat# 10933) recognized only one single band (54-Kd) in D283 cells (A). Following treatment of a specific SiRNA of Su(Fu), the endogenous Su(Fu) band was greatly reduced (B). Immunohistostaining with Su(Fu) antibodies in prostate cancer specimens revealed positive (C, in red, 200×), negative (D, 200×) or weak staining (E, red, 200×).

These data indicate that activation of the hedgehog pathway is quite common in prostate cancer. Tumors with activated hedgehog signaling either over-express the positive regulator Shh, or lack protein expression of the negative regulator Su(Fu).

Mutation analysis of Su(Fu) in prostate cancer

To confirm the immunohistochemistry data, we performed immunoblotting analyses using several dissected TURP (Transurethral resection of the prostate) specimens in which tumor portion can be as high as 70% of the tissue mass. As shown in Fig. 5A, two tumors (PC48 and PC51) had no detectable Su(Fu) protein, which are consistent with our immunohistostaining. The matched normal tissues, however, retained expression of Su(Fu), indicating that alteration of Su(Fu) is a somatic event. Sequence analyses of these two tumors revealed genetic mutations in *Su(Fu)*, which are predicted to create STOP codons in the coding sequence (Fig. 5B). In PC48, a homozygous deletion of A1315 was detected, which results in a STOP codon at +1318 bp (Fig. 5B). In PC51, we detected two types of mutations, one with a deletion of C255, which results in a STOP codon at +294 bp whereas another with a deletion of C198, create a STOP codon (Picture not shown here, see Table 1). These mutations were confirmed with 6 independent clones from two separate experiments, which exclude the possibility of PCR errors. No mutations were detected from the matched benign tissues, indicating the somatic nature of the mutations. Real-time PCR analyses indicated that target genes of the hedgehog pathway, PTCH1 and Gli1, were all elevated in these tumors (Fig. 5C), confirming activation of the hedgehog pathway in these tumors. Thus, Su(Fu) inactivation appears to contribute to activation of hedgehog signaling in these prostate tumors.

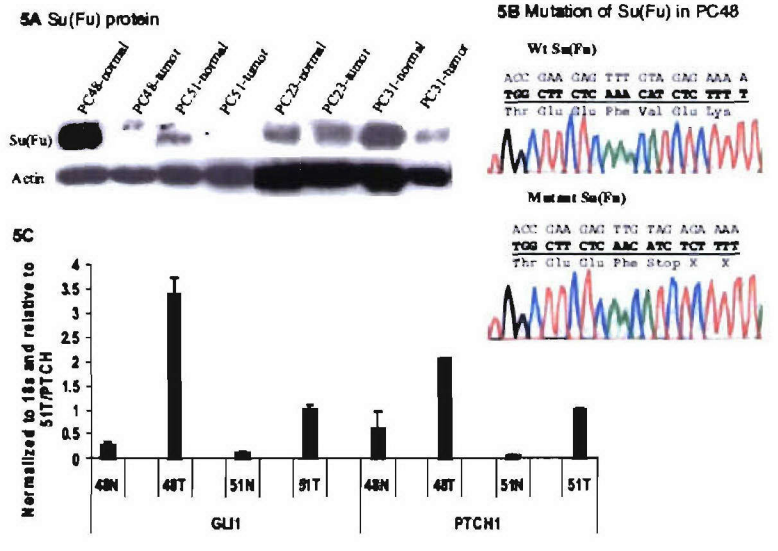


Fig. 5 Inactivation of Su(Fu) in prostate cancer. Two TURP specimens were examined for Su(Fu) protein by immunoblotting. Consistent with loss of Su(Fu) in the tissue, we did not detect Su(Fu) protein in these two tumors (PC48 and PC51 (5A). Further analysis revealed mutations of Su(Fu) in the tumor (PC48, 5B), which created a STOP codon in Su(Fu). We further found that tumor with mutant Su(Fu) had high levels of hedgehog target genes, PTCH1 and Gli1 (5C), indicatiing that hedgehog signaling is activated.

These data provide strong evidence that hedgehog signaling activation occurs in prostate cancer, through loss of Su(Fu) or over-expression of Shh.

Task 2:

Inhibition of hedgehog signaling for cell growth and cell invasiveness

Identification of Shh over-expression was unexpected in the proposal. Because of the availability of hedgehog signaling inhibitor, cyclopamine, the effects of hedgehog signaling activation on cell proliferation and invasiveness was demonstrated in several prostate cancer cell lines, in which Shh is over-expressed. In the presence of hedgehog signaling inhibitor, cyclopamine, both cell proliferation and cell invasiveness was dramatically reduced, indicating that the hedgehog pathway is required for both cell proliferation and cell invasiveness of prostate cancer cells.

Following treatment with 5 μM cyclopamine in PC3 cells, expression of hedgehog target genes were dramatically inhibited (data not shown here), which was accompanied with a significant reduction of BrdU positive cells (see Fig. 6B for details). This effect is specific because addition of tomatidine, a non specific compound with a similar structure to cyclopamine, had no effects on either target gene expression or DNA synthesis (indicated by BrdU labeling in Fig. 6B and 6C). The prostate epithelial RWPE-1 cells which have no activated hedgehog signaling, on the other hand, were not sensitive to cyclopamine (data not shown here), indicating that cyclopamine specifically affects cells with elevated hedgehog signaling. LN-CAP, Du145 and TSU cells, like PC3 cells were also sensitive to cyclopamine treatment (Fig. 6C).

Prostate cancer progression is accompanied by increased cell invasiveness. Because the hedgehog signaling activation occurs frequently in advanced prostate cancer, we examined if inhibition of the hedgehog signaling can reduce cell invasiveness. Using BD Bio-coat cell invasion chambers, we found that treatment of cyclopamine in PC3 cells reduced the percentage of invasive cells by 70% (Fig. 7A). Similar data were also observed in Du145, LN-CAP and TSU cells (Fig. 7B). Under the same condition, RWPE-1 cells were not very invasive. Thus, hedgehog signaling activation regulates both cell proliferation as well as cell invasiveness of prostate cancer cells.

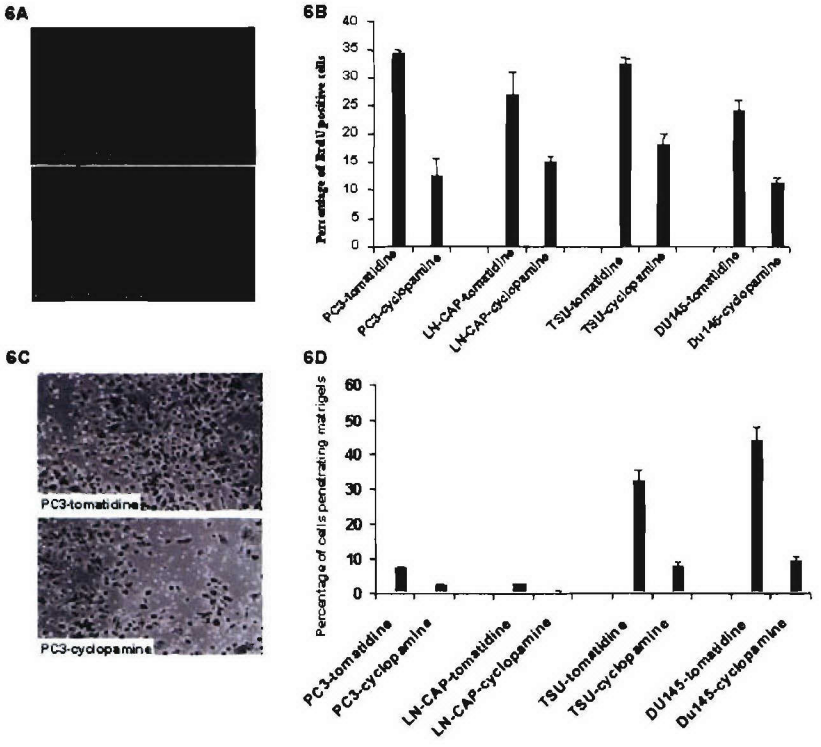


Fig. 6 The effects of targeted inhibition of hedgehog signaling on DNA synthesis and cell invasiveness DNA synthesis was detected by BrdU labeling (A). Over 1000 cells were counted under fluorescent microscope for the percentage of BrdU positive cells, and the experiment was repeated twice (B). Cell invasion assay of prostate cancer cells was performed using BD Bio-coat cell invasion chambers (C). The rate of cell invasion was calculated by dividing cell numbers penetrated the matrigels by the number of cells in the control chambers (without matrigels) (D).

The role of Su(Fu) on cell proliferation

On the other hand, the only cancer cell line with no detectable expression of Su(Fu) is a lung cancer cell line. We have demonstrated that re-expression of Su(Fu) slowed cell growth of this cell line, and reduced the percentage of S phase cell population (see Fig. 1). We are planning to establish inducible expression of Su(Fu) in this cell line to understand molecular details of hedgehog signaling activation triggered by loss of Su(Fu).

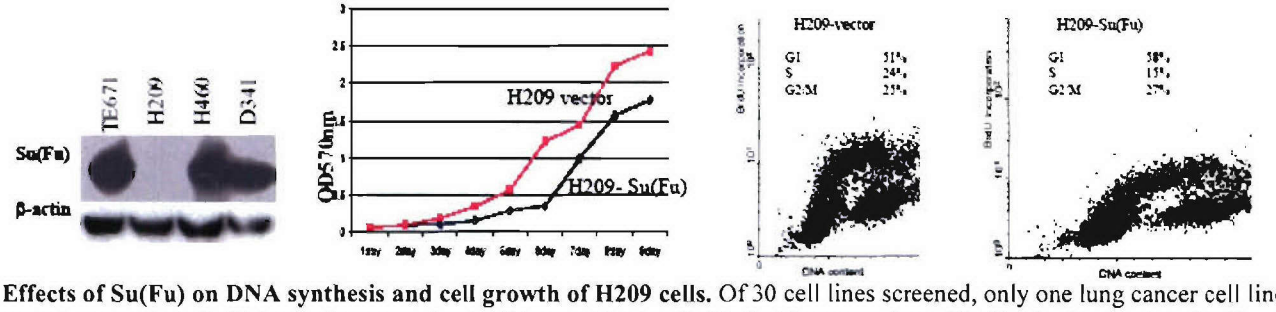


Fig. 7 Effects of Su(Fu) on DNA synthesis and cell growth of H209 cells. Of 30 cell lines screened, only one lung cancer cell line, H209, was shown to lack Su(Fu) protein expression by Western blotting (**left**). Stable expression of Su(Fu) leads to reduced cell growth (**middle**). In consistence with cell growth, Su(Fu) reduced the percentage of S phase cell population (**right**).

Because prostate cancer cell lines without Su(Fu) expression are not available, this part of the study has been slower than expected. Currently, we are trying to inactivate Su(Fu) in a normal prostate epithelial cell line, RWPE-1, using inducible expression of SiRNA. Our expectation is that loss of Su(Fu) in this cell line may lead to aggressive cell growth, and possibly tumor formation in nude mice.

Task 3: Currently, we are still trying to establish mice with Nkx3.1-/- ; Ptch1+/- mice. Hopefully, we can get some progress in the next few months. We will report the progress next year.

Key Research Accomplishments

With support from DOD, we have demonstrated that activation of the hedgehog pathway occurs in advanced and metastatic prostate cancers (Molecular Cancer 3: 29, 2004).Two other independent groups have also shown similar results (Nature 431: 707, 2004; PNAS 101: 12561, 2004). These findings indicate that inhibition of hedgehog signaling may be an effective approach in future treatments of prostate cancer.

Reportable outcomes

One research paper (Molecular Cancer 3: 29, 2004), one review paper and two news releases.

Conclusion

We have demonstrated that hedgehog pathway activation occurs frequently in advanced prostate cancer.

References

1. Sheng T, Li C, Zhang X, Chi S, He N, Chen K, et al. Activation of the hedgehog pathway in advanced prostate cancer. *Mol Cancer* 2004;3(1):29. Available from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15482598
2. Sanchez P, Hernandez AM, Stecca B, Kahler AJ, DeGueme AM, Barrett A, et al. Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling. *Proc Natl Acad Sci U S A* 2004;101(34):12561-6. Available from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15314219
3. Karhadkar SS, Bova GS, Abdallah N, Dhara S, Gardner D, Maitra A, et al. Hedgehog signalling in prostate regeneration, neoplasia and metastasis. *Nature* 2004;431(7009):707-12. Available from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15361885
4. Xie J. Hedgehog signaling in prostate cancer. *Future Oncology* 1(3): 331-338, 2005. http://www.futuremedicine.com/doi/pdfplus/10.1517/14796694.1.3.331

Appendices

1. Reprint of the Molecular Cancer 3:29, 2004.
2. Copy of the news release for prostate cancer research.
3. Copy of the news release for cancer treatment using cyclopamine.
4. Reprint of Future Oncology 1:331-338, 2005.

Molecular Cancer

BioMed Central

Research

Open Access

Activation of the hedgehog pathway in advanced prostate cancer

Tao Sheng[†1], Chengxin Li[†1,2], Xiaoli Zhang[†1], Sumin Chi[1,2], Nonggao He[1], Kai Chen[1], Frank McCormick[3], Zoran Gatalica[4] and Jingwu Xie[*1]

Address: [1]Sealy Centers for Cancer Cell Biology and Environmental Health, Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, Texas, 77555-1048, USA, [2]Department of Dermatology, Xijing hospital, Xi'an 710032, China, [3]UCSF Cancer Center, 2340 Sutter Street, San Francisco, CA 94115, USA and [4]Department of Pathology, Creighton University Medical Center, 601 N 30th St. Omaha, NE 68131, USA

Email: Tao Sheng - tasheng@utmb.edu; Chengxin Li - cxli66@yahoo.com; Xiaoli Zhang - xiaolzha@utmb.edu; Sumin Chi - chisumin@yahoo.com; Nonggao He - nhe@utmb.edu; Kai Chen - kachen@utmb.edu; Frank McCormick - mccormick@cc.ucsf.edu; Zoran Gatalica - zgatalica@pathology.creighton.edu; Jingwu Xie[*] - jinxie@utmb.edu

[*] Corresponding author [†]Equal contributors

Published: 13 October 2004

Molecular Cancer 2004, **3**:29 doi:10.1186/1476-4598-3-29

Received: 22 September 2004
Accepted: 13 October 2004

This article is available from: http://www.molecular-cancer.com/content/3/1/29

Abstract

Background: The hedgehog pathway plays a critical role in the development of prostate. However, the role of the hedgehog pathway in prostate cancer is not clear. Prostate cancer is the second most prevalent cause of cancer death in American men. Therefore, identification of novel therapeutic targets for prostate cancer has significant clinical implications.

Results: Here we report that activation of the hedgehog pathway occurs frequently in advanced human prostate cancer. We find that high levels of hedgehog target genes, PTCH1 and hedgehog-interacting protein (HIP), are detected in over 70% of prostate tumors with Gleason scores 8–10, but in only 22% of tumors with Gleason scores 3–6. Furthermore, four available metastatic tumors all have high expression of PTCH1 and HIP. To identify the mechanism of the hedgehog signaling activation, we examine expression of Su(Fu) protein, a negative regulator of the hedgehog pathway. We find that Su(Fu) protein is undetectable in 11 of 27 PTCH1 positive tumors, two of them contain somatic loss-of-function mutations of *Su(Fu)*. Furthermore, expression of sonic hedgehog protein is detected in majority of PTCH1 positive tumors (24 out of 27). High levels of hedgehog target genes are also detected in four prostate cancer cell lines (TSU, DU145, LN-Cap and PC3). We demonstrate that inhibition of hedgehog signaling by smoothened antagonist, cyclopamine, suppresses hedgehog signaling, down-regulates cell invasiveness and induces apoptosis. In addition, cancer cells expressing Gli1 under the CMV promoter are resistant to cyclopamine-mediated apoptosis. All these data suggest a significant role of the hedgehog pathway for cellular functions of prostate cancer cells.

Conclusion: Our data indicate that activation of the hedgehog pathway, through loss of Su(Fu) or overexpression of sonic hedgehog, may involve tumor progression and metastases of prostate cancer. Thus, targeted inhibition of hedgehog signaling may have significant implications of prostate cancer therapeutics.

Background

The *hedgehog* (Hh) pathway plays a critical role in embryonic development and tissue polarity [1]. Secreted Hh molecules bind to the receptor *patched* (PTC-PTCH1, PTCH2), thereby alleviating PTC-mediated suppression of *smoothened* (SMO), a putative seven-transmembrane protein. SMO signaling triggers a cascade of intracellular events, leading to activation of the pathway through GLI-dependent transcription [2]. The hedgehog receptor PTCH1 is also a target gene of this pathway, which forms a negative feedback mechanism to maintain the pathway activity at an appropriate level in a given cell. Activation of Hh signaling through loss-of-function somatic mutations of PTCH1 in human basal cell carcinomas (BCCs) disrupts this feedback regulation, leading to uncontrolled SMO signaling. Activating mutations of SMO in BCCs, on the other hand, are resistant to PTCH1-mediated inhibition, leading to an outcome similar to PTCH1 inactivation [3-6]. More recently, abnormal activation of the sonic hedgehog pathway, through over-expression of sonic hedgehog, has been implicated in the development of subsets of medulloblastomas, small cell lung cancer and gastrointestinal tract (GI) cancers [7-10].

Development of prostate requires hedgehog signaling. Although the initial formation of prostate buds does not require sonic hedgehog signaling (shh), shh is critical for maintaining appropriate prostate growth, proliferation and tissue polarity [11-14]. In the adult prostate, however, the activity of the hedgehog pathway is quite low. It remains to be tested whether this hedgehog pathway is activated during development of prostate cancer, the second most prevalent cause of cancer death in American men. Activation of the hedgehog pathway is often indicated by elevated levels of PTCH1 and HIP. In addition to PTCH1 mutation, SMO activation and hedgehog over-expression, loss of Su(Fu) can result in activation of the hedgehog pathway. In the human, the Su(Fu) gene is localized at chromosome 10q24, a region with LOH in several types of cancer including prostate cancer, lung cancer, breast cancer and medulloblastomas [15,16]. As a negative regulator of the hedgehog pathway, Su(Fu) inhibits the function of Gli molecules, leading to inactivation of this pathway [17-19]. Su(Fu) is also reported to affect beta-catenin function [20]. In addition, over-expression of sonic hedgehog is shown to be involved in the development of GI cancers [9,10]. Here we report our findings that activation of the hedgehog pathway occurs frequently in advanced prostate cancers, possibly through loss of Su(Fu) protein or over-expression of sonic hedgehog.

Results

Elevated expression of hedgehog target genes in prostate cancer specimens

As an important regulator of tissue polarity, active hedgehog signaling is required for ductual morphogenesis and proliferation during prostate development [11-14]. The adult prostate, on the other hand, does not contain active hedgehog signaling. Because hedgehog signaling is an important regulator for epithelial-mesenchymal interaction, an event critical during prostate cancer development, we examined whether the hedgehog-signaling pathway is activated in prostate cancer.

Activation of hedgehog signaling causes elevated expression of target genes PTCH1 and HIP. Thus, increased protein expression of PTCH1 and HIP indicates activation of the hedgehog pathway. Using PTCH1 antibodies [10], we examined 59 prostate cancer samples for hedgehog signaling activation (see Table 1, Additional file 1 for details). We first tested the specificity of the PTCH1 antibodies in MEF cells. *Ptch1* null MEF cells have no active *Ptch1* gene, thus should not have positive staining with PTCH1 antibodies. Indeed, no staining was seen in *Ptch1* null MEF cells (Fig. 1A). After transfection of *PTCH1* expressing plasmid, transfected cells showed positive staining (Fig. 1A), indicating that the PTCH1 antibodies are specific to PTCH1. Furthermore, PTCH1 immunohistostining was abolished after addition of the specific peptide, from which the antibodies were raised (Fig. 1B,1c). We found that percentage of PTCH1 positive staining tumors increased in high grade tumors (Table 1, Additional file 1). In prostate cancers with Gleason scores 3–6, 4 out of 18 specimens were positive for PTCH1 (22%), whereas 16 out of 22 undifferentiated carcinomas (Gleason Scores of 8–10) expressed PTCH1 (73%, see Table 1, Additional file 1), suggesting that the hedgehog pathway is frequently activated in advanced prostate cancer. To confirm this data, we found that all four available metastatic prostate cancer specimens were all positive for PTCH1 staining.

To further confirm our data, we detected HIP protein expression, another marker of the hedgehog signaling activation. After transfection of HIP expressing plasmid into 293 cells, HIP antibodies recognize a single band around 75 KD (Fig. 3A), and an endogenous HIP protein with a similar size was also detected in two cancer tissues, in which hedgehog signaling is known to be activated (Fig. 3B and data not shown here). In contrast, the matched normal tissue did not express detectable HIP. Thus, HIP expression appears to be a good marker for hedgehog signaling activation. Immunohistostaining with HIP antibodies in prostate cancer specimens revealed a similar pattern to prostate specific antigen (PSA) and PTCH1 (Fig. 3C and Table 1, Additional file 1), further confirming that hedgehog pathway is activated in

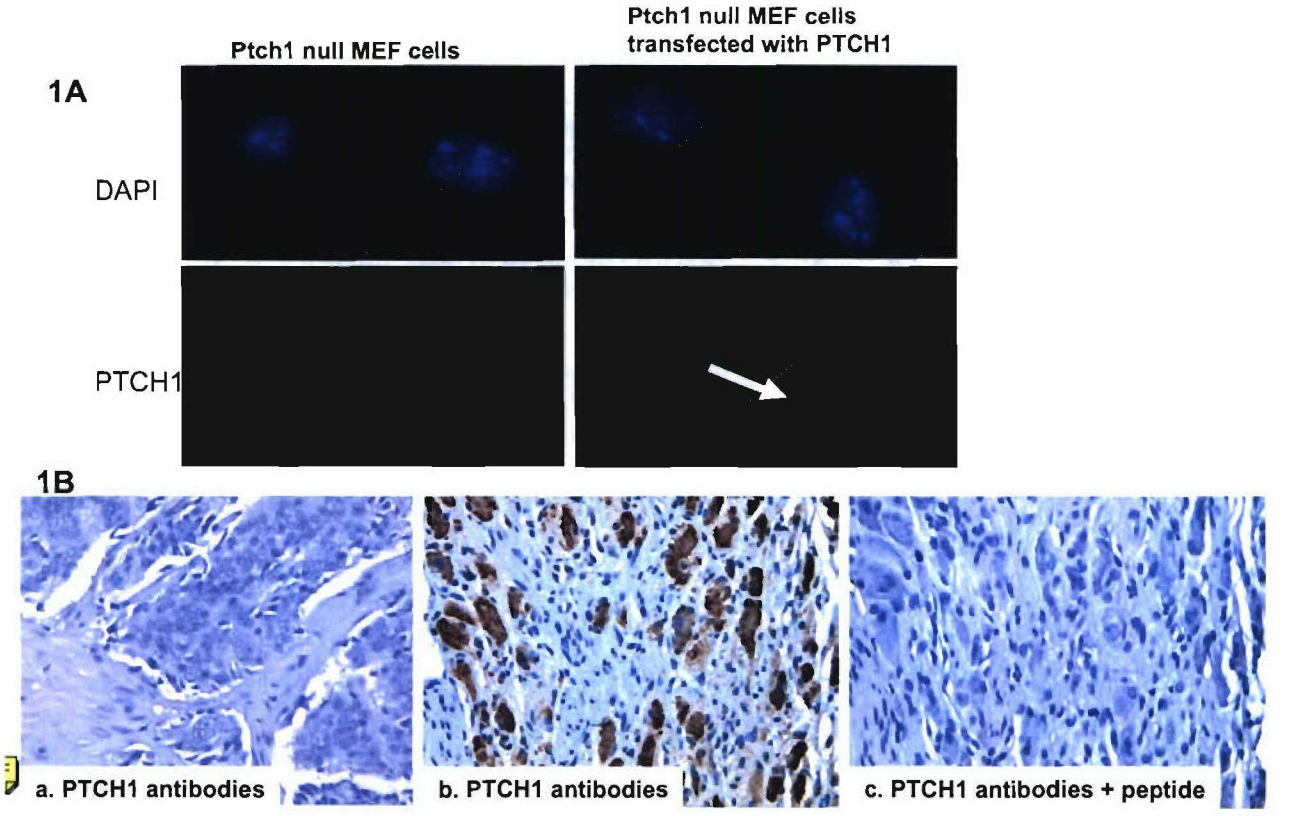


Figure 1

Detection of PTCH1 expression in prostate cancers. Protein expression of PTCH1 was detected by immunostaining. PTCH1 antibodies (Santa Cruz Biotechnology Cat# 9149) were tested in *Ptch1*$^{-/-}$ null MEF cells (**A**). While *Ptch1*$^{-/-}$ null MEF cells had no positive fluorescent staining with PTCH1 antibodies, transfection of PTCH1 expressing plasmid lead to positive staining (green, indicated by an arrow, 400×). Immunohistochemistry of prostate cancer specimens with PTCH1 gave negative (**B-a,** 200×) or positive (Red in **B-b,** 200×) signals. When PTCH1 antibodies were pre-incubated with the very peptide for raising the antibodies, no positive signals could be observed (**B-c**).

advanced prostate cancers. Thus the hedgehog pathway appears to be frequently activated in advanced or metastatic prostate cancers.

Altered expression of Su(Fu) and Shh in prostate cancer specimens

There are several mechanisms by which the hedgehog pathway in these prostate tumors can be activated, including loss of Su(Fu) or over-expression of hedgehog [6-10]. The Su(Fu) gene is localized at 10q24, a region with a frequent LOH in prostate cancer [15,16,18]. Mutations of Su(Fu) have been reported in other human cancers [6]. To test whether loss of Su(Fu) function is responsible for hedgehog signaling activation, we examined expression of Su(Fu) protein in these prostate cancer specimens. The antibodies of Su(Fu) recognize a single band at 52-kD in Western blotting analyses (Fig. 4A), which was reduced following treatment with Su(Fu) SiRNA (Fig. 4B), indicating the specificity of the antibodies. Furthermore, addition of the peptide, from which the antibodies were raised, prevented the antibody binding, further confirming the specificity of our Su(Fu) antibodies (data not shown). Of the 16 PTCH1 positive prostate cancer specimens with Gleason scores 8–10, 9 have no detectable Su(Fu) protein (Fig. 4C,4D,4E and Table 1, Additional file 1). In total, 11 of 27 PTCH1 positive prostate cancer specimens have no detectable Su(Fu) protein. Prostate cancers with low Gleason scores, however, frequently have

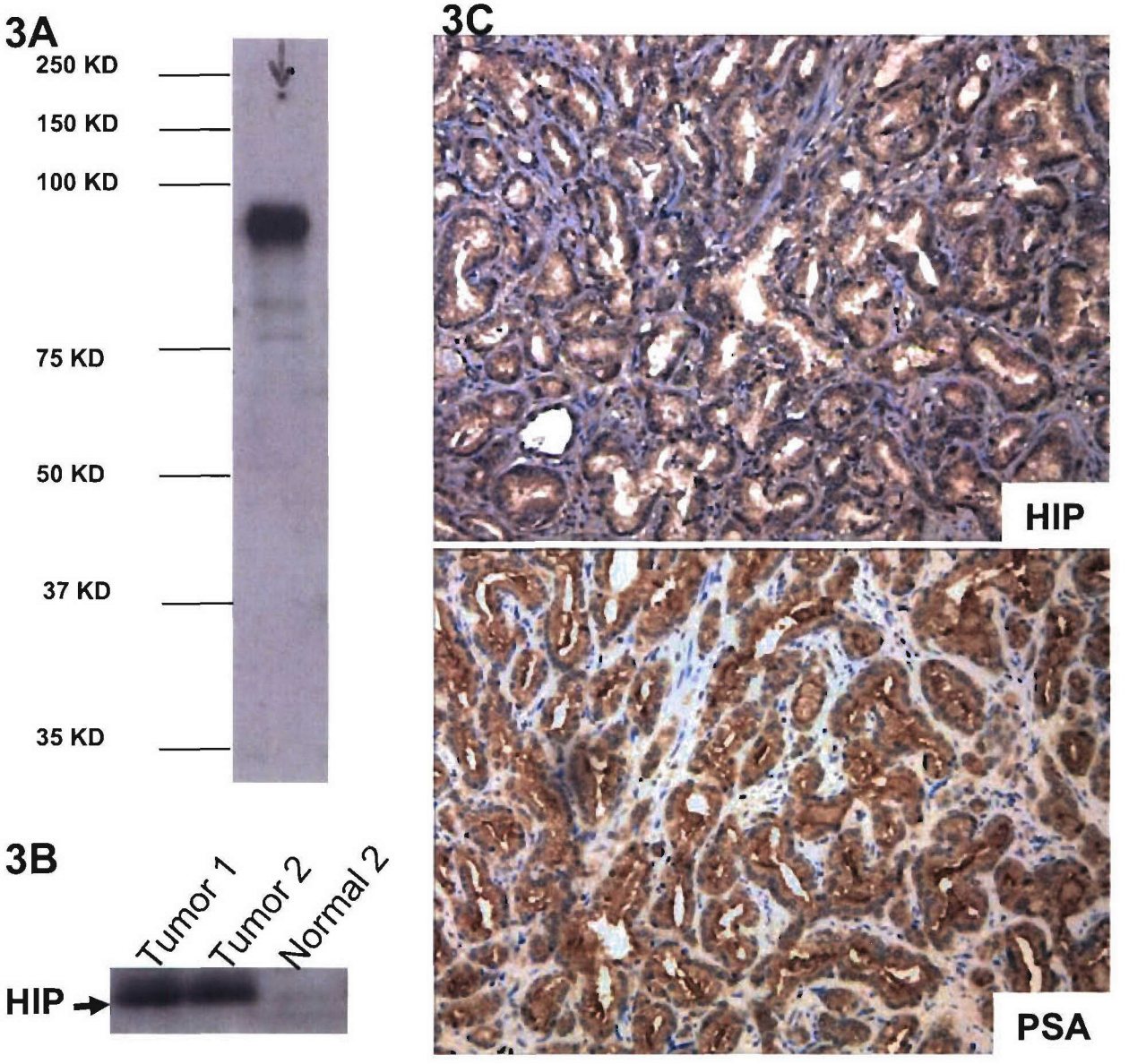


Figure 3
Detection of HIP in human cancer specimens. By Western blotting, HIP antibodies (R&D systems Cat# AF1568) recognized one band between 75 and100 KD (**A**). Expression of endogenous HIP was detected in two GI cancer tissues, which were known to contain activated hedgehog signaling (data not shown here), but not in the matched normal tissue (**B**). Immunohistostaining of HIP I prostate cancer showed a similar pattern to PSA (**C**, 200×)

detectable Su(Fu) protein (see Table 1, Additional file 1), suggesting that loss of Su(Fu) protein may be associated with prostate cancer progression.

To confirm the immunohistochemistry data, we performed immunoblotting analyses using several dissected TURP (Transurethral resection of the prostate) specimens in which tumor portion can be as high as 70% of the tissue mass. Prostatectomy specimens (most of our tumors), however, often contain a small percentage (5–10%) of tumor tissue and are therefore not suitable for Western blotting or real-time PCR analyses. As shown in Fig. 5A,

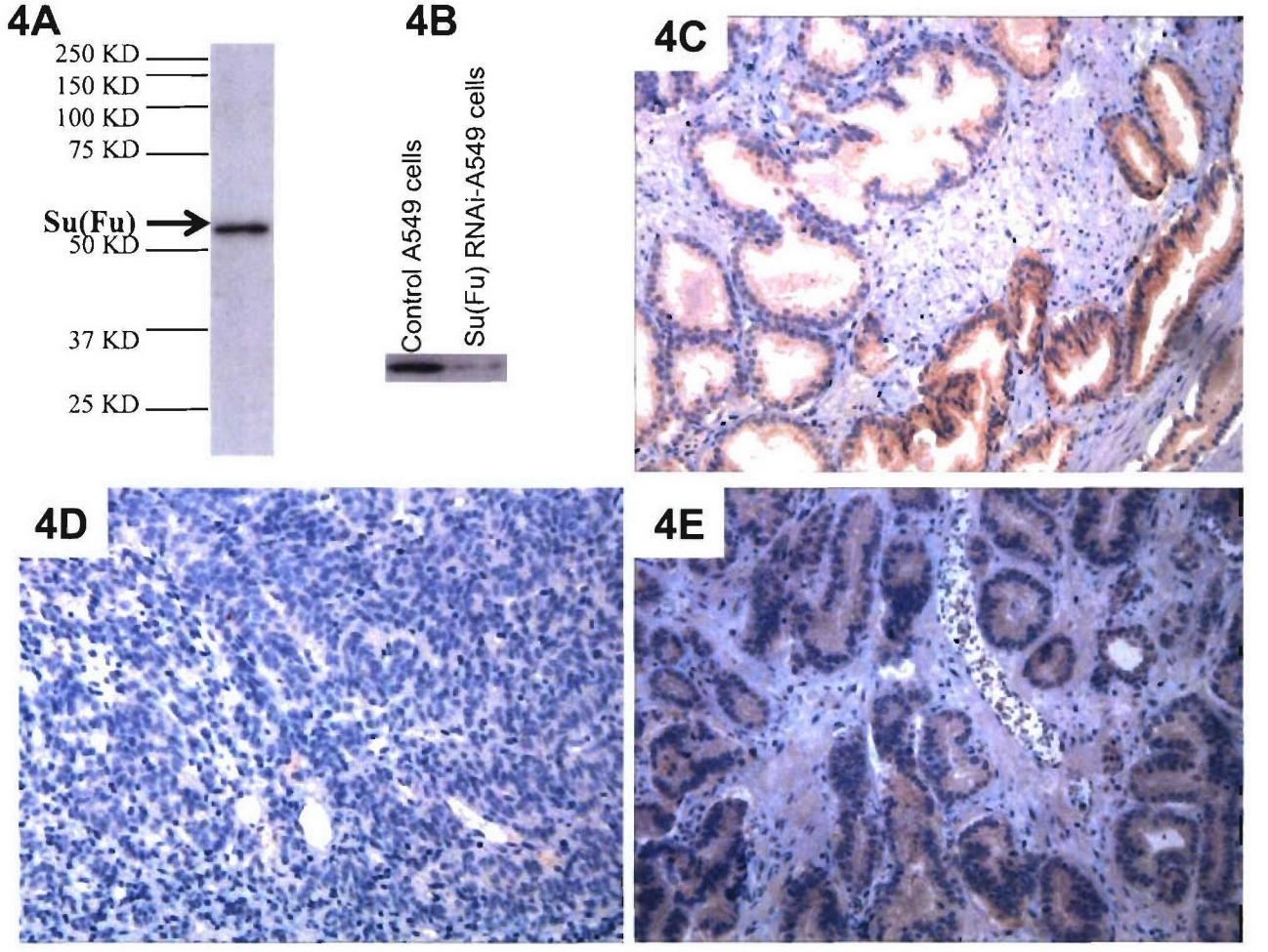


Figure 4

Detection of Su(Fu) in prostate cancer specimens. Su(Fu) antibodies (Santa Cruz Biotechnology Cat# 10933) recognized only one single band (54-Kd) in D283 cells (**A**). Following treatment of a specific SiRNA of Su(Fu), the endogenous Su(Fu) band was greatly reduced (**B**). Immunohistostaining with Su(Fu) antibodies in prostate cancer specimens revealed positive (**C**, in red, 200×), negative (**D**, 200×) or weak staining (**E**, red, 200×).

two tumors (PC48 and PC51) had no detectable Su(Fu) protein, which are consistent with our immunohistostaining, suggesting loss of Su(Fu) may be responsible for hedgehog pathway activation in these tumors. The matched normal tissues, however, retained expression of Su(Fu), indicating that alteration of Su(Fu) is a somatic event. Sequence analyses of these two tumors revealed genetic mutations in *Su(Fu)*, which are predicted to create STOP codons in the coding sequence (Fig. 5B and Table 1, Additional file 1). In PC48, a homozygous deletion of A1315 was detected, which results in a STOP codon at +1318 bp (Fig. 5B). In PC51, we detected two types of mutations, one with a deletion of C255, which results in a STOP codon at +294 bp whereas another with a deletion of C198, create a STOP codon (Picture not shown here, see Table 1, Additional file 1). These mutations were confirmed with 6 independent clones from two separate experiments, which exclude the possibility of PCR errors. No mutations were detected from the matched benign tissues, indicating the somatic nature of the mutations. Real-time PCR analyses indicated that target genes of the hedgehog pathway, PTCH1 and Gli1, were all elevated in these tumors (Fig. 5C), confirming activation of the hedgehog pathway in these tumors. Thus, Su(Fu) inactivation appears to contribute to activation of hedgehog signaling in these prostate tumors.

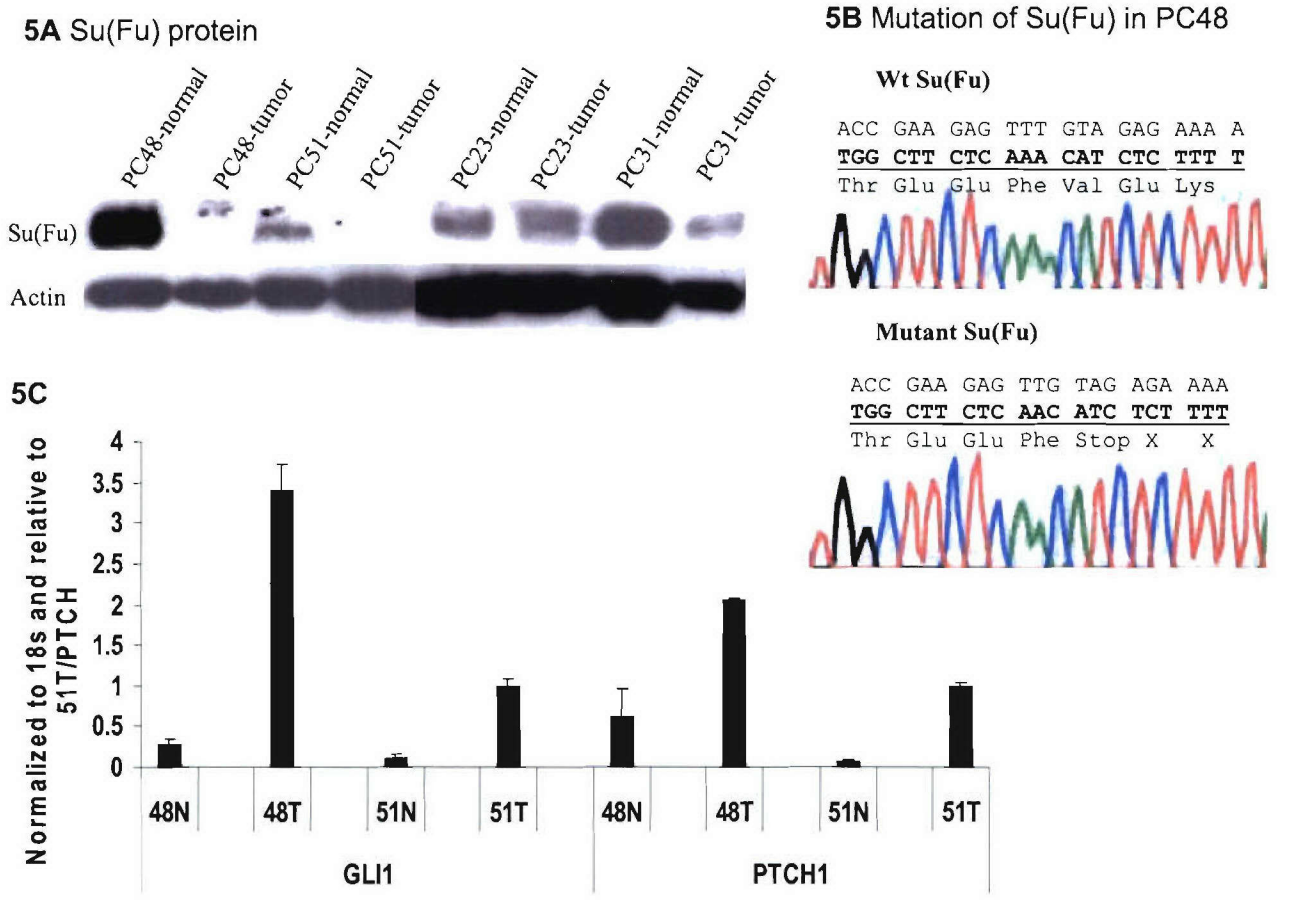


Figure 5

Inactivation of Su(Fu) in prostate cancer. Two TURP (Transurethral resection of the prostate) tumors with loss of Su(Fu) expression were confirmed by Western blotting (**A**). One mutation of Su(Fu) found in prostate cancer PC48 is shown in **B**, which is predicted to create a STOP codon in the Su(Fu) coding sequence +1318. The levels of Gli1 and PTCH1 transcripts in prostate tissues were detected by real-time PCR (see methods for details) (**C**). Tumor tissues had higher levels of the target gene transcripts.

For tumors with high level of PTCH1 expression, but no changes in Su(Fu) protein expression, we examined expression of sonic hedgehog. It is reported that expression of hedgehog may be responsible for hedgehog signaling activation in lung cancer and GI cancers. Immunohistostaining with sonic hedgehog antibodies indicate that sonic hedgehog is highly expressed in 24 of 27 advanced prostate tumors with elevated expression of PTCH1 and HIP (see Fig. 2 and Table 1, Additional file 1). Thus, activation of the hedgehog pathway, as indicated by elevated PTCH1 and HIP expression, is associated with loss of Su(Fu) expression or elevated hedgehog expression.

The role for activated hedgehog signaling for cellular functions of prostate cancer

To demonstrate the role of hedgehog pathway in prostate cancer, we screen five available cell lines for the expression of Gli1, PTCH1 and HIP. TSU, LNCap, Du145 and PC3 are prostate cancer cell lines whereas RWPE-1 is a prostate epithelial cell line. We found that the hedgehog target genes were significantly elevated in all cancer cell lines (Fig. 6A). Thus, we predicted that inhibition of the hedgehog pathway by smoothened antagonist, cyclopamine, would suppress cell proliferation and cell invasiveness.

Following treatment with 5 μM cycloapmine in PC3 cells, expression of hedgehog target genes were dramatically inhibited (data not shown here), which was accompanied

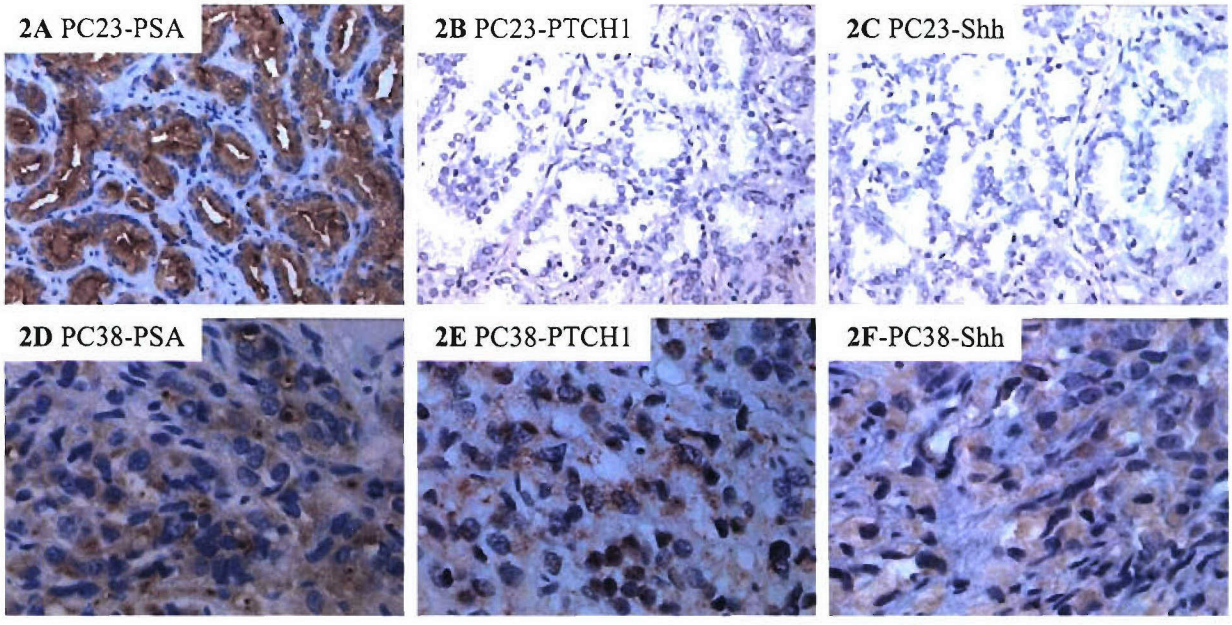


Figure 2
Co-expression of PTCH1, PSA and Shh in prostate cancer specimens. Immunohistochemistry of prostate cancer specimens with PSA was used to confirm the cancer region. Positive staining was in red. Positive staining patterns of PTCH1 and Shh antibodies (Santa Cruz Biotechnology Cat# 9024) were similar to that of PSA staining. PC23 (**A-C**) was from tumors with Gleason score 7 (200×). PC38 (**D-F**) was a tumor from Gleason score 10 (400×) (**see** Table 1, Additional file 1 for details).

with a significant reduction of BrdU positive cells (see Fig. 6B for details). This effect is specific because addition of tomotidine, a non specific compound with a similar structure to cycloapmine, had no effects on either target gene expression or DNA synthesis (indicated by BrdU labeling in Fig. 6B and 6C). The prostate epithelial RWPE-1 cells which have no activated hedgehog signaling, on the other hand, were not sensitive to cyclopamine (data not shown here), indicating that cyclopamine specifically affects cells with elevated hedgehog signaling. LN-CAP, Du145 and TSU cells, like PC3 cells were also sensitive to cyclopamine treatment (Fig. 6C).

Prostate cancer progression is accompanied by increased cell invasiveness. Because the hedgehog signaling activation occurs frequently in advanced prostate cancer, we examined if inhibition of the hedgehog signaling can reduce cell invasiveness. Using BD Bio-coat cell invasion chambers, we found that treatment of cyclopamine in PC3 cells reduced the percentage of invasive cells by 70% (Fig. 7A). Similar data were also observed in Du145, LN-CAP and TSU cells (Fig. 7B). Under the same condition, RWPE-1 cells were not very invasive. Thus, hedgehog signaling activation regulates both cell proliferation as well as cell invasiveness of prostate cancer cells.

It has been shown that cyclopamine induced apoptosis in cancer cells with activated hedgehog signaling [21]. We have shown that Gli1 down-regulation is necessary for cyclopamine-mediated apoptosis in basal cell carcinoma cells [21]. To test the significant role of Gli1, the downstream effector and the target gene of the hedgehog pathway, in cyclopamine-mediated apoptosis, we first transfected Gli1 expressing plasmid in to PC3 cells, and then treated the cells with 5 μM cyclopamine for 36 h. Since Gli1 is expressed under the control of the CMV promoter, we predicted that ectopic Gli1-expressing cells should be resistant to apoptosis, which is detected by TUNEL staining. As shown in Fig. 8, we found that all Gli1 positive cells (n = 500) were TUNEL negative, supporting our hypothesis that down-regulation of Gli1 may be an important mechanism by which cyclopamine mediates apoptosis in prostate cancer cells with activated hedgehog signaling.

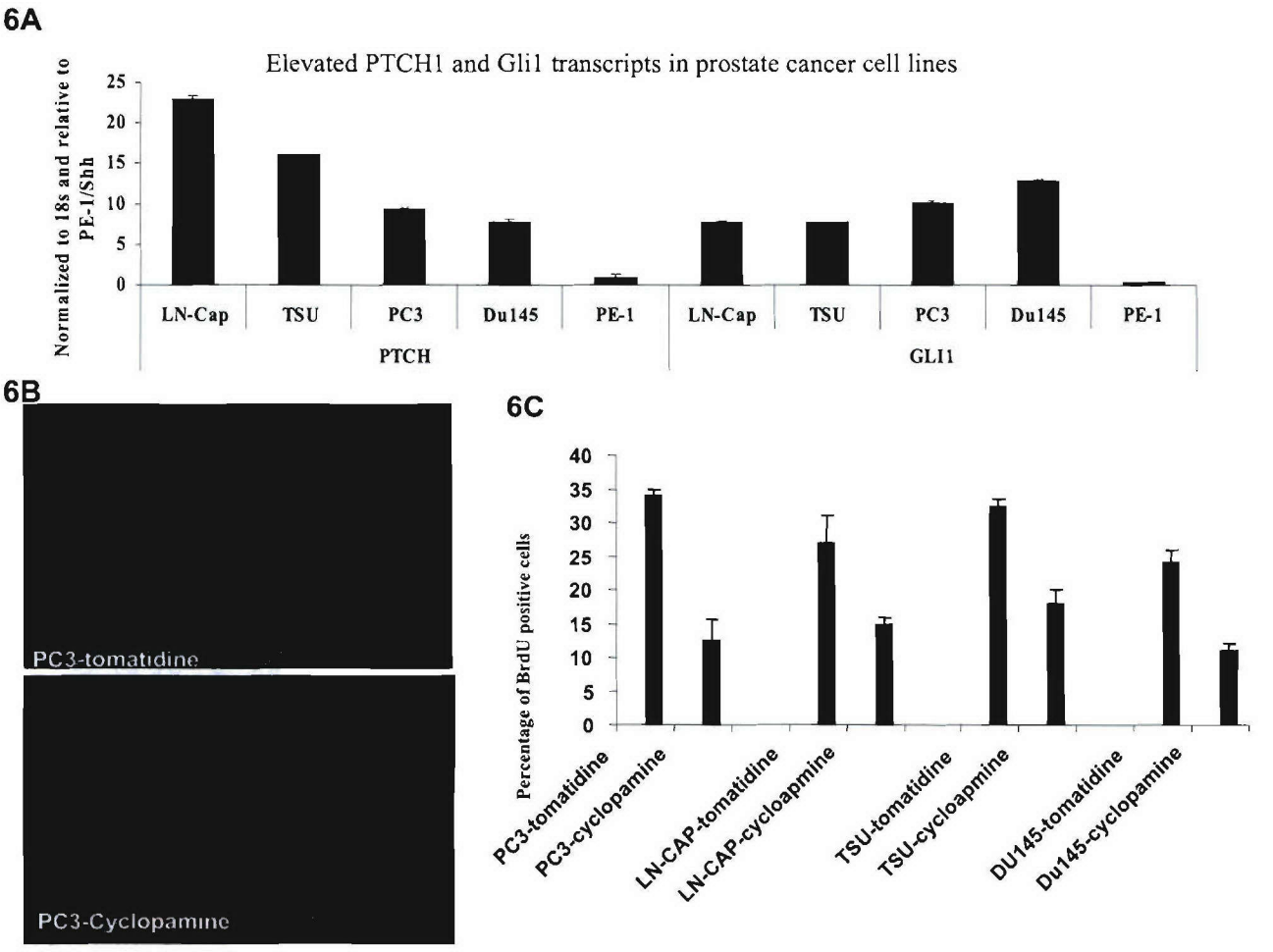


Figure 6
Cellular functions of the hedgehog pathway in prostate cancer cells. Expression of hedgehog target genes, PTCH1 and Gli1, were detected by real-time PCR (**A**). DNA synthesis was detected by BrdU labeling (**B**). Over 1000 cells were counted under fluorescent microscope for the percentage of BrdU positive cells, and the experiment was repeated twice (**C**).

All these data indicate that the hedgehog pathway is activated in advanced prostate cancers, as indicated by high expression of PTCH1 and HIP. Our results also indicate that hedgehog signaling is required for cell proliferation and cell invasion of prostate cancer cells. Thus, targeted inhibition of the hedgehog pathway may be effective in future prostate cancer therapeutics.

Discussion

Hedgehog signaling pathway regulates cell proliferation, tissue polarity and cell differentiation during normal development. Abnormal signaling of this pathway has been reported in a variety of human cancers, including basal cell carcinomas, medulloblastomas, small cell lung cancer and GI cancers [3,4,6-10,22,23]. Our findings in this report indicate a role of the sonic hedgehog pathway in prostate cancer. We detected a high expression of hedgehog target genes, PTCH1 and HIP, in advanced or metastatic prostate cancers. In contrast, only 22% of prostate tumors with Gleason scores 3-6 have elevated expression of PTCH1 and HIP. While our manuscript is being reviewed, three independent groups have recently reported similar results [24-26]. Thus, the hedgehog signaling pathway is frequently activated in advanced or metastatic prostate cancers.

Alterations of genes in the hedgehog pathway in prostate cancer

In our studies, we found that some prostate tumors had no detectable Su(Fu) protein expression while others

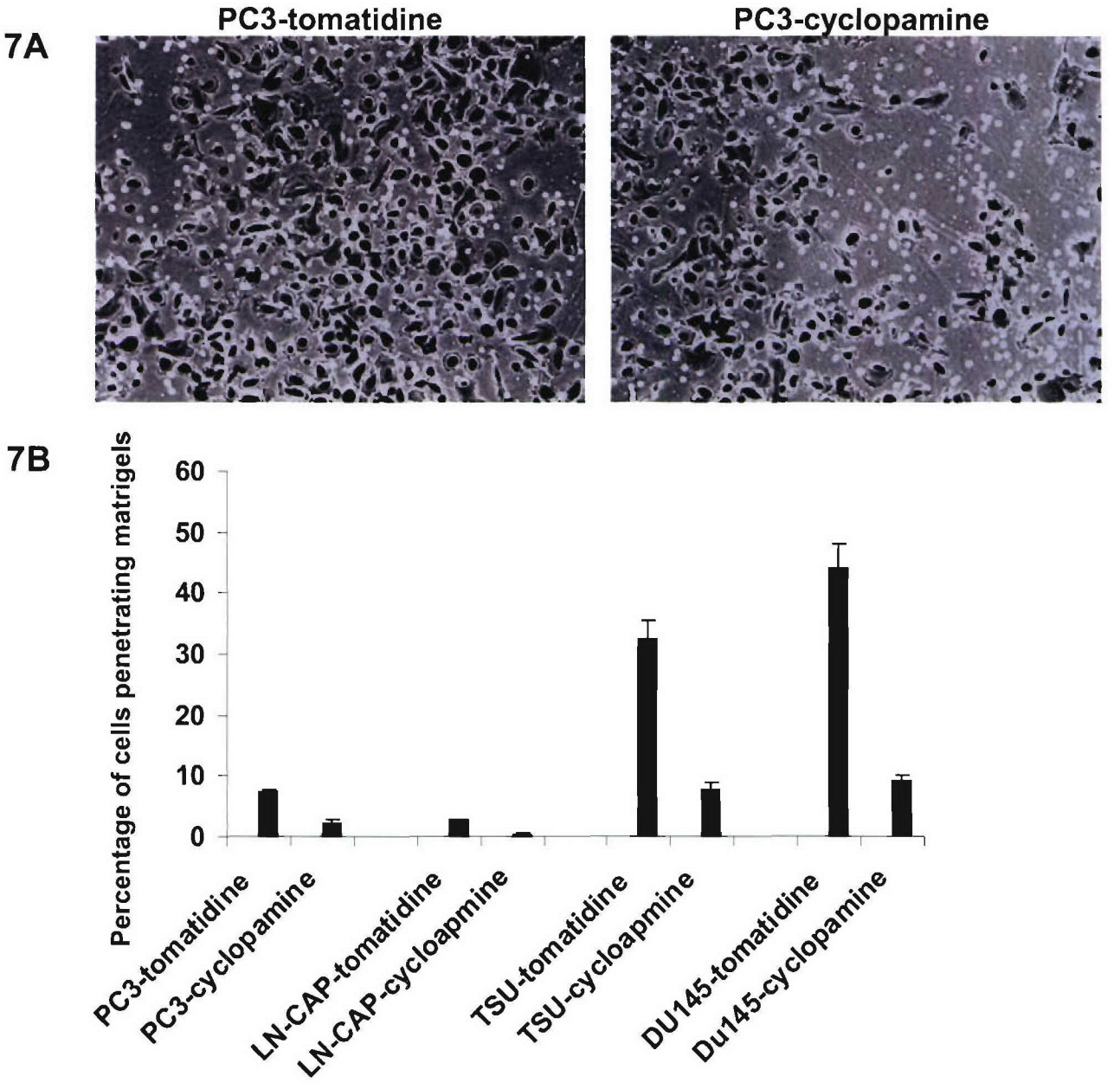


Figure 7
Effects of cyclopamine on cell invasiveness of prostate cancer cells. Cell invasion assay of prostate cancer cells was performed using BD Bio-coat cell invasion chambers (**A**). The rate of cell invasion was calculated by dividing cell numbers penetrated the matrigels by the number of cell in the control chambers (without matrigels) (**B**).

contained high levels of Shh protein expression. We further identified inactivated mutations of *Su(Fu)* in two prostate cancers. In addition to inactivated mutations in the coding region, Su(Fu) may be inactivated through promoter methylation. The heterogeneous nature of prostate cancer makes it difficult to screen prostate cancer specimens for Su(Fu) mutations since the tumor content is often less than 5% of the specimens. Future improve-

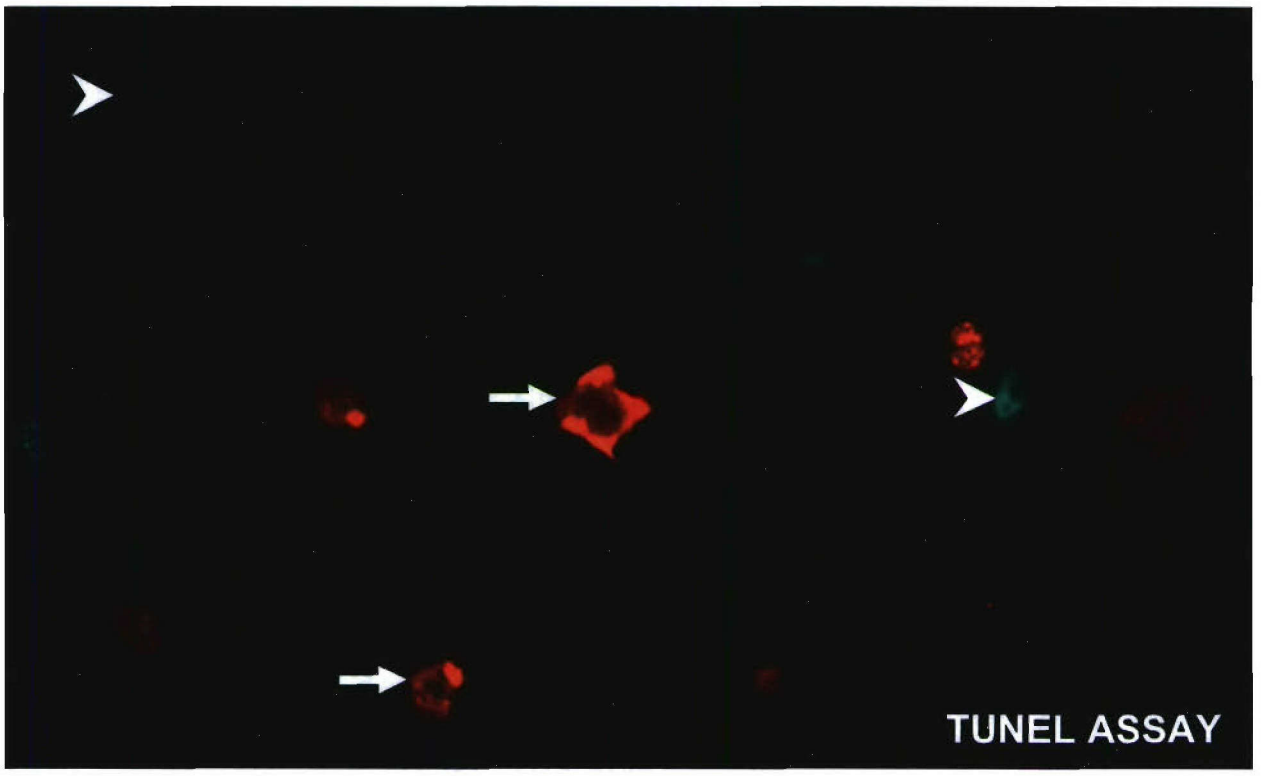


Figure 8
Cyclopamine induces apoptosis in prostate cancer cells. Cyclopamine-mediated apoptosis in prostate cancer cells was analyzed by TUNEL assay. TUNEL positive cells were indicated by arrowheads. Cells with expression of Gli1 under the CMV promoter (indicated by the arrows) did not undergo apoptosis (n = 500).

ment can be achieved using microdissection techniques for collecting pure population of tumor cells in gene mutation analysis.

Since all available prostate cancer cell lines express Su(Fu) at a high level, the role of Su(Fu) on cellular functions of prostate cancer cannot be investigated in these cells. It appears that over-expression of sonic hedgehog may be responsible for hedgehog signaling activation in these cell lines [our unpublished data and [24-26]]. After screening over 30 human cancer cell lines, we identified non-prostate cancer cell line with elevated hedgehog target genes and no detectable Su(Fu) expression (data not shown here). The growth suppression effects of Su(Fu) was demonstrated in this cell line, in which Su(Fu) expression down-regulated hedgehog target genes, inhibited DNA synthesis and cell growth (data not shown here). Thus, inactivation of Su(Fu) can contribute to active hedgehog signaling in prostate cancer.

Su(Fu) is reported to affect β-catenin signaling [27,28]. We analyzed expression of β-catenin and E-cadherin in our prostate cancer array and detected cytoplasmic distribution of E-cadherin and β-catenin only in PC51 (data not shown), indicating that Su(Fu) may be able to affect both the wnt pathway and the hedgehog pathway in prostate cancer.

In addition to Su(Fu) inactivation, over-expression of Shh is another mechanism by which the hedgehog pathway is activated in cancer [7-10]. We noticed that sonic hedgehog expression varies from tumor to tumor, which may be resulted from the heterogeneity of prostate cancer. Our immunohistostaining also revealed that three tumors (PC14, PC20 and PC36) expressed PTCH1 and HIP at high levels, but had no alterations of Shh and Su(Fu). This could be due to elevated expression of indian hedgehog, or even alterations of other components of the pathway (such as Rab23 or Fused).

Once hedgehog pathway is activated, the target gene expression will be up-regulated. Thus, analysis of target gene expression using immunohistochemistry will be an effective way to detect hedgehog pathway activation in prostate cancer. Currently, PTCH1, Gli1 and HIP are good markers for the hedgehog pathway.

Perspectives on prostate cancer therapy

Our findings not only provide novel basic understanding of prostate cancer, but also allow us to design new ways to treat prostate cancer. With a specific SMO antagonist, cyclopamine, it will be possible in the future to treat prostate cancers, which have over-expressed sonic hedgehog. However, as a downstream molecule, tumors with Su(Fu) inactivation may not respond to cyclopamine treatment. Therefore, additional small molecule inhibitors appear to be necessary to treat Su(Fu) inactivated prostate cancer. One possibility is to use Gli1 SiRNA since we have indicated that down-regulation of Gli1 may be an important mechanism by which inhibition of the hedgehog pathway by cyclopamine induces apoptosis (Fig. 8). Sanchez et al also indicated that Gli1 SiRNA down-regulated DNA synthesis in prostate cancer cells [24].

Conclusion

Taken together, our findings suggest that activation of the hedgehog pathway involves prostate cancer progression. There might be several mechanisms by which the hedgehog pathway is activated in advanced prostate cancers, including loss of Su(Fu) protein expression, over-expression of sonic hedgehog or other alterations. We demonstrate that activation of the hedgehog pathway is associated with DNA synthesis and cell invasiveness in prostate cancer cells. Inhibition of the hedgehog pathway, on the other hand, causes apoptosis possibly through down-regulation of Gli1. Our studies predict that targeted inhibition of the hedgehog pathway may be an effective way to prevent prostate cancer progression.

Materials and methods

Tissue Microarray of Prostate Cancer

A total of 55 paraffin-embedded tissue blocks from patients with prostate cancer were obtained from UTMB Surgical pathology with approval from the Institutional Review Board (IRB). Pathological reports, H#E staining of each specimen were reviewed to determine the nature of the disease and the Gleason scores. Of 55 specimens, 18 were from tumors with Gleason scores 3–6, 15 with Gleason score 7 and 22 with Gleason scores 8–10. The tumor area was first identified before tissue microarray (1.5 mm in diameter for specimens) was assembled with Beecher's Tissue arrayer-I® according to manufacturer's instruction http://www.beecherinstruments.com.

Immunohistochemistry and Western blotting

A standard avidin-biotin immunostaining technique was performed using a kit from Vector laboratories using specific antibodies to Su(Fu) (Santa Cruz Biotechnology Cat# 10933), PTCH1 (Santa Cruz Biotechnology Cat# 6149), HIP (R&D systems Cat# AF1568) and Shh (Santa Cruz Biotechnology Cat# 9024) and PSA (Vector laboratories). Positive staining was in red or brown. The specificity of antibodies was tested using the very peptide used for raising the antibodies, which abolished the specific staining. Hematoxylin was used for counterstaining (in blue). Protein was analyzed by Western analysis with appropriate antibodies [Su(Fu) antibodies were from Santa Cruz, beta-actin antibody was purchased from Sigma). The signals were visualized with the enhanced chemiluminescence detection system (Amersham).

Cell lines and Cell invasion assay

Cell lines (RWPE-1, Du145, PC3, LN-CAP were purchase from ATCC and cultured according to the suggested conditions. TSU was kindly provided by Dr. Allen Gao. Cell invasion assay was performed with BD Bio-coat cell invasion chambers according to manufacturer's instruction (BD Bioscience, Inc., Franklin Lakes, NJ), with triplicates for each sample and the experiment was repeated three times with the similar results. Cell were treated with 5 μM cyclopamine (or tomatidine) before (for 12 h) and during cell invasion assay (for 24 h). The rate of cell invasion was calculated by dividing cell numbers penetrated the matrigels by the number of cell in the control chambers (without matrigels).

RT-PCR and sequencing analysis

Total RNA was isolated using Trizol® reagent (Invitrogen), and RT-PCR was performed using Promega's RT-PCR system according to the manufacturer's protocol. Two pairs of Su(Fu) primers were used (the first set with the forward primer 5'-cctacgcaccccgatggcg-3" and the reverse primer 5'-agccaaaaccactacctcca-3'; the second set with the forward primer 5'-tccaggttaccgctatcgtc-3' ad the reverse primer 5'-tagtgtagcggactgtcg-3'). PCR products were first purified using Qiagen's Gel Extraction Kit. Due to existence of possible Su(Fu) splicing isoforms in humans, Su(Fu) genetic mutations were screened after the PCR products were cloned into TOPO® TA cloning vectors (Invitrogen). Several independent clones (from three experiments) of each PCR product were selected for sequencing analysis in UTMB sequencing facility. All mutations were confirmed by at least six independent clones.

Real-time PCR We used Applied Biosystems' assays-by-demand 20× assay mix of primers and TaqMan probes (FAM™ dye-labeled) for the target genes (human Gli and PTCH1, the sequences have been patented by Applied Biosystems, Foster City, CA) and pre-developed 18S rRNA

(VIC™-dye labled probe) TaqMan® assay reagent (P/N 4319413E) for an internal control. The primers are designed to span exon-exon junctions so as not to detect genomic DNA and the primers and probe sequences were searched against the Celera database to confirm specificity. To obtain the relative quantitation of gene expression, a validation experiment was performed to test the efficiency of the target amplification and the efficiency of the reference amplification. All absolute values of the slope of log input amount vs. ΔC_T were <0.1. Separate tubes (singleplex) one-step RT-PCR was performed with 20 ng RNA for both target genes and endogenous control. The reagent we used was TaqMan one-step RT-PCR master mix reagent kit (P/N 4309169). The cycling parameters for one-step RT-PCR was: reverse transcription 48°C for 30 min, AmpliTaq activation 95°C for 10 min, denaturation 95°C for 15 sec and annealing/extension 60°C for 1 min (repeat 40 times) on ABI7000. Triplicate C_T values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer(Applied Biosystems, Foster City, CA). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalization to an endogenous reference (18sRNA) and relative to a calibrator.

BrdU labeling and TUNEL assay

BrdU labeling was performed using an *in situ* cell proliferation kit (Roche Molecular Biochemicals) [22]. Cells were treated with 5μM cyclopamine (or tomatidine) for 12 h before BrdU labeling (1 h at 37°C). The percentage of BrdU positive cells was obtained by counting over 1000 cells under microscope, and the experiment was repeated twice with similar results. TUNEL assay was performed using an *in situ* cell death kit (Roche Molecular Biochemicals) [21,29]. Cells were treated with 5 μM cyclopamine (or tomatidine) for 36 h before TUNEL assay).

List of abbreviations

PSA – prostate specific antigen; HIP – hedgehog-interacting protein; Su(Fu) – suppressor of fused; PTCH1 – human homologue of *patched* 1; Shh – sonic hedgehog; SMO – smoothened, BCC – basal cell carcinoma.

Authors' contributions

Tao Sheng contributed to Figures 6, 7, 8, cellular functions of the hedgehog pathway in prostate cancer cells. Chegxin Li contributed to primary tumor protein expression, particularly on Su(Fu) expression. Xiaoli Zhang contributed to mutation analyses of Su(Fu) in prostate cancer and real-time PCR analyses. Sumin Chi contributed to HIP antibody test (Fig. 3A and 3B). Nonggao He contributed to HIP antibody staining (Fig. 3C). Kai Chen contributed to PTCH1 antibody test (Fig. 1A). Frank McCormick involved in the initial project discussion. Zoran Gatalica contributed to prostate cancer histology and Gleason scores of the tumors.

Additional material

Additional File 1

Table 1 Prostate cancer specimens and protein expression. Prostate cancer specimens and expression of several hedgehog signaling proteins are summarized in this table (A). A total of 55 specimens were used in this study. The Gleason scores and protein expression of Shh, PTCH1 and Su(Fu) are shown (B).

Click here for file

[http://www.biomedcentral.com/content/supplementary/1476-4598-3-29-S1.doc]

Acknowledgements

we thank Dr. Huiping Guo for technical support of real-time PCR. J.X. was supported by grants from a NIH R01 grant, a DOD grant and the Sealy Foundation for biomedical Sciences.

References

1. Ingham PW: **Transducing Hedgehog: the story so far.** *Embo J* 1998, **17**:3505-3511.
2. Taipale J, Beachy PA: **The Hedgehog and Wnt signalling pathways in cancer.** *Nature* 2001, **411**:349-354.
3. Johnson RL, Rothman AL, Xie J, Goodrich LV, Bare JW, Bonifas JM, Quinn AG, Myers RM, Cox DR, Epstein E. H., Jr., Scott MP: **Human homolog of patched, a candidate gene for the basal cell nevus syndrome.** *Science* 1996, **272**:1668-1671.
4. Hahn H, Wicking C, Zaphiropoulous PG, Gailani MR, Shanley S, Chidambaram A, Vorechovsky I, Holmberg E, Unden AB, Gillies S, Negus K, Smyth I, Pressman C, Leffell DJ, Gerrard B, Goldstein AM, Dean M, Toftgard R, Chenevix-Trench G, Wainwright B, Bale AE: **Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome.** *Cell* 1996, **85**:841-851.
5. Xie J, Murone M, Luoh SM, Ryan A, Gu Q, Zhang C, Bonifas JM, Lam CW, Hynes M, Goddard A, Rosenthal A, Epstein E. H., Jr., de Sauvage FJ: **Activating Smoothened mutations in sporadic basal-cell carcinoma.** *Nature* 1998, **391**:90-92.
6. Taylor MD, Liu L, Raffel C, Hui CC, Mainprize TG, Zhang X, Agatep R, Chiappa S, Gao L, Lowrance A, Hao A, Goldstein AM, Stavrou T, Scherer SW, Dura WT, Wainwright B, Squire JA, Rutka JT, Hogg D: **Mutations in SUFU predispose to medulloblastoma.** *Nat Genet* 2002, **31**:306-310.
7. Berman DM, Karhadkar SS, Hallahan AR, Pritchard JI, Eberhart CG, Watkins DN, Chen JK, Cooper MK, Taipale J, Olson JM, Beachy PA: **Medulloblastoma growth inhibition by hedgehog pathway blockade.** *Science* 2002, **297**:1559-1561.
8. Watkins DN, Berman DM, Burkholder SG, Wang B, Beachy PA, Baylin SB: **Hedgehog signalling within airway epithelial progenitors and in small- cell lung cancer.** *Nature* 2003, **422**:313-317.
9. Berman DM, Karhadkar SS, Maitra A, Montes De Oca R, Gerstenblith MR, Briggs K, Parker AR, Shimada Y, Eshleman JR, Watkins DN, Beachy PA: **Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours.** *Nature* 2003, **425**:846-851.
10. Thayer SP, Di Magliano MP, Heiser PW, Nielsen CM, Roberts DJ, Lauwers GY, Qi YP, Gysin S, Fernandez-Del Castillo C, Yajnik V, Antoniu B, McMahon M, Warshaw AL, Hebrok M: **Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis.** *Nature* 2003, **425**:851-856.
11. Berman DM, Desai N, Wang X, Karhadkar SS, Reynon M, Abate-Shen C, et al.: **Roles for Hedgehog signaling in androgen production and prostate ductal morphogenesis.** *Dev Biol* 2004, **267**:: 387-398.

12. Podlasek CA, Barnett DH, Clemens JQ, Bak PM, Bushman W: **Prostate development requires Sonic hedgehog expressed by the urogenital sinus epithelium.** *Dev Biol* 1999, **209**:28-39.
13. Wang BE, Shou J, Ross S, Koeppen H, De Sauvage FJ, Gao WQ: **Inhibition of epithelial ductal branching in the prostate by sonic hedgehog is indirectly mediated by stromal cells.** *J Biol Chem* 2003, **278**:18506-18513.
14. Freestone SH, Marker P, Grace OC, Tomlinson DC, Cunha GR, Harnden P and Thomson AA.: **Sonic hedgehog regulates prostatic growth and epithelial differentiation.** *Dev Biol* 2003, **264**:: 352-362.
15. Latini JM, Rieger-Christ KM, Wang DS, Silverman ML, Libertino JA, Summerhayes IC: **Loss of heterozygosity and microsatellite instability at chromosomal sites 1Q and 10Q in morphologically distinct regions of late stage prostate lesions.** *J Urol* 2001, **166**:1931-1936.
16. Leube B, Drechsler M, Muhlmann K, Schafer R, Schulz WA, Santourlidis S, Anastasiadis A, Ackermann R, Visakorpi T, Muller W, Royer-Pokora B: **Refined mapping of allele loss at chromosome 10q23-26 in prostate cancer.** *Prostate* 2002, **50**:135-144.
17. Ding Q, Fukami S, Meng X, Nishizaki Y, Zhang X, Sasaki H, et al.: **Mouse suppressor of fused is a negative regulator of sonic hedgehog signaling and alters the subcellular distribution of Gli1.** *Curr Biol* 1999, **9**:1119-1122.
18. Kogerman P, Grimm T, Kogerman L, Krause D, Unden AB, Sandstedt B, et al.: **Mammalian suppressor-of-fused modulates nuclear-cytoplasmic shuttling of Gli-1.** *Nat Cell Biol* 1999, **1**:312-319.
19. Stone DM, Murone M, Luoh S, Ye W, Armanini MP, Gurney A, Phillips H, Brush J, Goddard A, de Sauvage FJ, Rosenthal A: **Characterization of the human suppressor of fused, a negative regulator of the zinc-finger transcription factor Gli.** *J Cell Sci* 1999, **112**:4437-4448.
20. Meng X, Poon R, Zhang X, Cheah A, Ding Q, Hui CC, Alman B: **Suppressor of fused negatively regulates beta-catenin signaling.** *J Biol Chem* 2001, **276**:40113-40119.
21. Athar M, Li CX, Chi S, Tang X, Zhang X, Kim AL, Tyring SK, Kopelovich L, Epstein EH Jr, Blickers DR, Xie J: **Inhibition of smoothened signaling prevents ultraviolet B-induced basal cell carcinomas through induction of fas expression and apoptosis.** *Cancer Res* 2004, **64**:7545-7552.
22. Xie J, Aszterbaum M, Zhang X, Bonifas JM, Zachary C, Epstein E, McCormick F: **A role of PDGFRalpha in basal cell carcinoma proliferation.** *Proc Natl Acad Sci U S A* 2001, **98**:9255-9259.
23. Xie J, Johnson RL, Zhang X, Bare JW, Waldman FM, Cogen PH, Menon AG, Warren RS, Chen LC, Scott MP, Epstein E. H., Jr.: **Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors.** *Cancer Res* 1997, **57**:2369-2372.
24. Sanchez P, Hernandez AM, Stecca B, Kahler AJ, DeGueme AM, Barrett A, et al.: **Inhibition of prostate cancer proliferation by interference with SONIC HEDGEHOG-GLI1 signaling.** *Proc Natl Acad Sci U S A* 2004, **101**:12561-12566.
25. Karhadkar SS, Steven Bova G, Abdallah N, Dhara S, Gardner D, Maitra A, et al.: **Hedgehog signalling in prostate regeneration, neoplasia and metastasis.** *Nature* 2004, **431**:707-712.
26. Fan L, Pepicelli CV, Dibble CC, Catbagan W, Zarycki JL, Laciak R, et al.: **Hedgehog signaling promotes prostate xenograft tumor growth.** *Endocrinology* 2004, **145**:3961-3970.
27. Meng X, Poon R, Zhang X, Cheah A, Ding Q, Hui CC and Alman B.: **Suppressor of fused negatively regulates beta-catenin signaling.** *J Biol Chem* 2001, **276**:40113-40119.
28. Taylor MD, Zhang X, Liu L, Hui CC, Mainprize TG, Scherer SW, et al.: **Failure of a medulloblastoma-derived mutant of SUFU to suppress WNT signaling.** *Oncogene* 2004, **23**:4577-4583.
29. Li C, Chi S, He N, Zhang X, Guicherit O, Wagner R, Tyring S, Xie J: **IFNalpha induces Fas expression and apoptosis in hedgehog pathway activated BCC cells through inhibiting Ras-Erk signaling.** *Oncogene* 2004, **23**:1608-1617.

Jim Kelly
(409) 772-NEWS
jpkelly@utmb.edu
FOR RELEASE: October 15, 2004

Contact UTMB Public Affairs

Media Hotline
(409)-772-6397

Front Desk
(409) 772-2618

public.affairs@utmb.edu

Plant-derived compound may treat and prevent the most common human cancer

GALVESTON, Texas—Researchers at the University of Texas Medical Branch at Galveston (UTMB) have demonstrated in laboratory animals that a compound derived from a common plant can be used to treat and prevent the most widespread form of cancer in people—the first such therapy to succeed in an experimental animal and one that may lead to therapies in people for this and other frequently encountered cancers.

The scientists report in the Oct. 15, 2004, issue of Cancer Research that oral administration of cyclopamine dramatically reduced tumor development in mice genetically engineered to be prone to the skin cancer known as basal cell carcinoma (BCC) when exposed to ultraviolet light. Cyclopamine is derived from the corn lily, a weed-like plant that grows in mountain meadows in the western United States.

The most common of all human cancers, BCC afflicts 800,000 Americans each year, according to the Skin Cancer Foundation. One of every three new cancers is a skin cancer, it says, and the vast majority are basal cell carcinomas.

"We showed 90 percent fewer microscopic BCC tumors after treating with cyclopamine, and 50 percent fewer visible tumors," said the study's lead author, Jingwu Xie, an assistant professor of pharmacology and toxicology at UTMB and scientist in the university's Sealy Center for Cancer Cell Biology. "Based on the microscopic tumor results, we see a potential to prevent new tumors from developing, while the visible tumor reduction shows us that this can be used for treatment. And since there's no noticeable toxicity to mice, this therapy has great clinical promise."

Because cyclopamine kills tumor cells by breaking only a single link in the chain of biochemical reactions leading to cancer— known to scientists as the "hedgehog pathway"— it should have far fewer side effects than more traditional chemotherapies, Xie said. ("Hedgehog" is a signaling protein important to animal growth and development that takes its name from the spiky appearance of fruit flies that lack the gene to produce it.) The hedgehog pathway has also been implicated in prostate cancer, brain tumors, lung cancer and some types of breast cancer, leading Xie and other researchers to believe that a compound like cyclopamine that shuts down hedgehog could serve as a weapon against many different kinds of cancer. (A paper by Xie examining the role of the hedgehog pathway in prostate cancer and describing the ability of cyclopamine to kill prostate cancer cells in test-tube experiments appeared this week in the online journal Molecular Cancer and can be found at http://www.molecular-cancer.com/home/.)

According to Xie, cyclopamine's safety and effectiveness against BCC tumors when given orally boost hopes that it could find wider clinical applications. "We used

drinking water to deliver the drug to the mice, and from drinking water to the circulatory system to the skin there are a lot of barriers," Xie said. "If you can treat skin tumors with an orally administered drug, you should be able to treat other kinds of tumor, too — gastrointestinal tumors, prostate cancers, lung cancers and some breast cancers."

The UTMB team collaborated on the research with scientists from Columbia University, the National Cancer Institute in Bethesda, Md., the University of California at San Francisco and the University of Texas Health Science Center at Houston .The paper, "Inhibition of Smoothened Signaling Prevents Ultraviolet B-induced Basal Cell Carcinomas through Regulation of Fas Expression and Apoptosis," can be found online starting Oct. 15 at http://cancerres.aacrjournals.org/.

-UTMB Newsroom-

Department of Defense awards UTMB Researchers $1.1 Million to Combat Prostate Cancer

The University of Texas Medical Branch at Galveston
Public Affairs Office
301 University Boulevard, Suite 3.102
Galveston, Texas 77555-0144
(409) 772-2618 / (800) 228-1841
www.utmb.edu

Tom Curtis
(409) 772-NEWS
tcurtis@utmb.edu
FOR RELEASE: June 18, 2004

GALVESTON, Texas—Three University of Texas Medical Branch at Galveston (UTMB) faculty members have won separate federal research awards for proposals that may lead to new strategies to prevent, detect and treat prostate cancer. Prostate cancer is the second most common malignancy in men after skin cancer, with an estimated 170,000 to 200,000 new cases annually in the United States alone and 30,000 U.S. deaths each year.

The three awards, totaling $1,124,648, were won by UTMB scientists Rinat O. Esenaliev, Owen P. Hamill and Jingwu Xie and were announced by the donor agency, the Department of Defense Prostate Cancer Research Program.

Esenaliev's research proposal — titled "Prostate Cancer Therapy with Novel Drug Delivery Technique"— puts forward a new way of selectively delivering any anti-cancer drug into prostate tumors without damage to normal tissues surrounding the tumors. Using mice bearing human prostate tumors, it employs ultrasound with small particles (nanoparticles) that selectively accumulate in the prostate tumor blood vessels. The ultrasound radiation combined with the drug-bearing nanoparticles induces formation, growth and collapse of tiny bubbles inside only the tumors, destroying the tumors and sparing surrounding tissues. The award to Esenaliev, an associate professor of neuroscience and cell biology and scientist with UTMB's Center for Biomedical Engineering, is $566,250. Hamill's proposal focuses on ultimately slowing or stopping the cell migration that helps spread prostate cancer tumor cells to sites elsewhere in the body, usually to the bone. An associate professor of neuroscience and cell biology, he theorizes that identifying and characterizing the molecules that enhance or suppress cell migration should lead to new ways to measure disease progression to treat prostate cancer. Titled "The Stretch-Activated CA2+-Permeable Channel: A Mechanosensory Switch for Invasiveness of Prostate Tumor Cells," Hamill's research proposal garnered an award of $113,250.

Xie, an assistant professor of pharmacology and toxicology and scientist with UTMB's Sealy Center for Cancer Cell Biology, observes that only a minority of prostate cancer tumors rapidly progress to become advanced cancers — the main cause of prostate cancer-related death. For that reason, he aims to identify the genetic signaling pathways enhancing or retarding cancer progression to help design strategies to combat prostate cancer. Specifically, Xie notes that two areas on a specific chromosome region ranging from 10q23 to 10q26 are commonly missing in those advanced prostate cancer, raising the possibility that their absence may facilitate development of prostate cancer. One area is known to be the home of a tumor suppressor gene called PTEN, which is found at 10q23. The other genes typically missing in those with prostate cancer haven't yet been identified, but Xie suggests that another gene called Su(Fu), found at chromosme region 10q24 to 10q25, is also a tumor suppressor. He hypothesizes that Su(Fu) forestalls development of prostate cancer by blocking activation of what is known as the "sonic hedgehog" pathway. Sonic hedgehog is a signaling protein essential to cellular functions such as tissue differentiation and cell proliferation. (It is named for a video game character because of the similar spiky appearance of skin cells with mutations in the genes that produce it.) In response to his grant of $566,148, Xie and his colleagues are (1) determining how

frequently the sonic hedgehog pathway is activated in advanced prostate cancer; (2) using cell lines to determine the mechanism by which Su(Fu) exerts its tumor-suppressing effects; and (3) using genetically engineered mice to determine the role of the sonic hedgehog pathway in promoting progression of prostate cancer.

--UTMB--

Hedgehog signaling in prostate cancer

Jingwu Xie

University of Texas Medical Branch at Galveston, Sealy Centers for Cancer Cell Biology and Environmental Health, Department of Pharmacology and Toxicology, 301 University Blvd, Galveston, TX 77555-1048, USA
Tel.: +1 409 747 1935; Fax: +1 409 747 1938; jinxie@utmb.edu

Prostate cancer is the most common malignancy and the second leading cancer-related cause of death in men in the USA. Despite enormous efforts in understanding the molecular basis of prostate cancer, very little progress has been made in prevention and treatment of this often lethal cancer. Recent studies have demonstrated that hedgehog signaling is frequently activated in advanced or metastatic prostate cancers. With small molecule inhibitors available to analyze the hedgehog signaling pathway, a novel rationale for prostate cancer therapy can be devised.

Keywords: basal cell carcinoma, cyclopamine, prostate specific antigen, patched gene, small interfering RNA, suppressor of fused, smoothened, sonic hedgehog, transurethral resection of the prostate

future medicine

Prostate cancer is the most common malignancy and the second leading cancer-related cause of death in men in the USA [1]. Aging is an important risk factor for prostate cancer, as over 70% of all prostate cancer cases are diagnosed in men over the age of 65 years. In addition, the incidence of prostate cancer in African–American men is significantly higher than that in Caucasian men. The most common method to diagnose prostate cancer is a measurement of the serum level of prostate-specific antigen (PSA). PSA screening has contributed to the increased 5-year survival rate of men diagnosed with prostate cancer. Radical prostatectomy is an effective treatment strategy for early prostate cancer; however, treatments for metastatic prostate cancer are generally not effective. For example, hormone therapy only temporarily reduces tumor size and, often, the tumor relapses a few years later due to androgen-independent tumor growth. Currently, little progress has been made in the prevention of prostate cancer [1]. Improved progress in these clinical areas will require a more detailed understanding of prostate cancer progression at the molecular level.

Development of prostate cancer is a multistep process, from precursor lesions, to invasive carcinoma, to metastatic tumor [2]. Gleason grading is the most commonly used system to define the aggressiveness of prostate cancer [3]. A high Gleason score suggests an aggressive tumor. Currently, the molecular alterations which accompany these morphologic changes remain poorly defined.

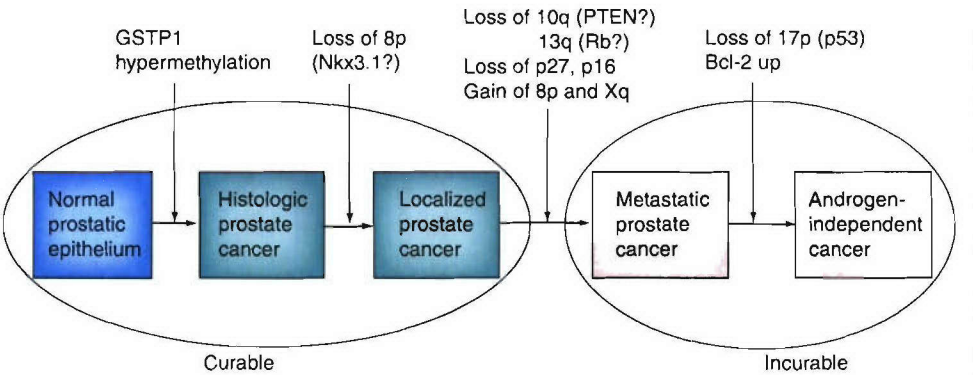
Cytogenetic and allelic loss studies have identified a number of chromosome regions potentially involved in prostate cancer development and progression [4,5] (Figure 1). Despite the significance of allelic loss for prostate carcinogenesis, no single tumor suppressor gene has been definitively assigned a role in cancer progression. The tumor suppressor protein phosphatase and tensin homolog deleted on chromosome ten (*PTEN*) has proven to be one of the best candidate tumor suppressors for prostate cancer [6]. In addition to genetic data, studies utilizing either clinical human specimens or mouse models have provided evidence of other molecules involving prostate cancer initiation and progression. Quantitative methylation-specific polymerase chain reaction (PCR) of the glutathione-*S*-transferase P1 (*GSTP1*) promoter has demonstrated near perfect specificity for cancer detection in prostate biopsies, and these studies indicate that *GSTP1* promoter hypermethylation is one of the earliest biomarkers for prostate cancer [7]. Loss of $p27^{kip1}$ is another biomarker for prostate cancer [8]. The role of *p27* in prostate cancer progression is further supported by targeted gene disruption in mice, which results in hyperplasia of multiple tissues including the prostate, and by the synergism between *p27* losses with PTEN inactivation [9]. Overexpression of Bcl-2 is an important marker of advanced, hormone-refractory prostate cancer, and may be responsible for the resistance of late-stage tumors to apoptosis [8]. Recent studies indicate a role of the hedgehog pathway in the development of prostate cancer and will serve as the focus of this review.

Hedgehog signaling in human cancers

The hedgehog pathway, initially identified in the fruit fly, is a master regulator of cell proliferation, tissue differentiation and tissue polarity. The hedgehog (Hh) pathway plays a critical role in embryonic development and tissue polarity. Activation of this pathway occurs frequently in human cancer, whereas inactivation of this pathway is associated with developmental disorders.

The current understanding of the hedgehog pathway will be discussed (Figure 2). There are

Figure 1. Major genetic alterations during prostate cancer progression.



Several genetic alterations are reported in prostate cancer. Methylation of the GSTP1 promoter and loss of chromosome 8p (containing the *Nkx3.1* gene) are the early changes. Loss of 10q [containing the PTEN gene], 13q (containing the *Rb* gene), loss of p27, p16 and gain of 8p and Xq are frequently found in advanced prostate cancers. Loss of chromosome 17p (containing the *p53* gene) and elevated *Bcl-2* expression are markers for metastatic prostate cancers.
GSTP1: Glutathione-*S*-transferase P1; PTEN: Phosphatase and tensin homolog deleted on chromosome ten; Rb: Retinoblastoma.

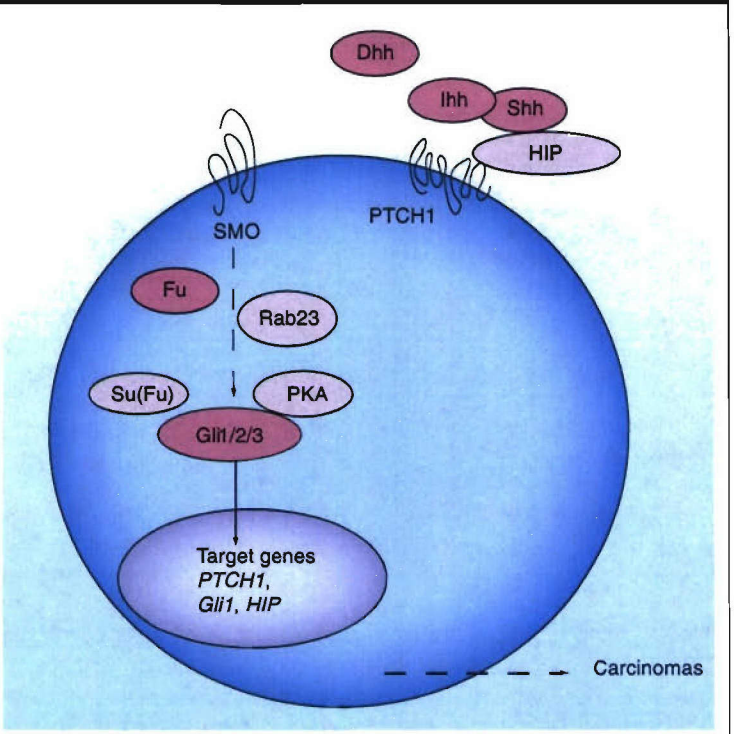
three distinct hedgehog genes in humans, sonic hedgehog, desert hedgehog and Indian hedgehog). These hedgehog proteins undergo similar post-translational modifications [10]. Secreted Hh molecules bind to the receptor patched (PTCH), thereby alleviating PTC-mediated suppression of smoothened (SMO). Expression of sonic hedgehog (Shh) appears to stabilize SMO protein possibly through post-translational modification of SMO. This effect of hedgehog molecules can be inhibited by hedgehog-interacting protein (HIP) through competitive association with PTC [11]. In *Drosophila*, SMO stabilization triggers complex formation with Costal-2, Fused and Gli homolog *Cubitus interruptus* (CI), which prevents CI degradation and formation of a transcriptional repressor. However, such a mechanism has not been established in mammalian cells. SMO ultimately activates transcription factors of the Gli family. There is genetic evidence indicating that several proteins link SMO to Gli. These signal transducers include Fu, suppressor of fused (Su[Fu]), Rab23 and protein kinase A (PKA) [12]. As transcriptional factors, Gli molecules can regulate target gene expression by directly associating with a consensus binding site (5′-TTTGGTTGCA-3′) located in the promoter region of target genes.

There are several regulatory feedback loops in this pathway (Figure 2). *PTC*, *Gli1* and *HIP*, which are components of this pathway, are also the target genes. PTC and HIP provide a negative feedback mechanism to maintain the pathway activity at an appropriate level in a given cell. In contrast, Gli1 forms a positive regulatory loop. Alteration of these loops will result in abnormal signaling of the hedgehog pathway.

Studies on hedgehog signaling are accelerated by availability of specific small molecular inhibitor cyclopamine, a natural product from *Veratrum californicum*, the corn lily [13]. Several synthetic compounds, which can promote or inhibit SMO signaling, are also available [14,15].

The link between the hedgehog pathway and human cancer was first identified in the Gorlin syndrome, a disease with a high risk of basal cell carcinomas (BCCs), medulloblastomata and meningiomas [16,17]. Loss-of-function mutations of the patched gene (*PTCH*)*1* are the cause of Gorlin syndrome. BCC, the most common human cancer, consistently has abnormalities of the hedgehog pathway and has often lost the function of *PTCH1* via point mutations and loss of the remaining allele. Most *PTCH1* mutations lead to loss of the protein function. Elevated expression of *Gli1* is an important indicator of this pathway activation in BCCs [18]. Mice heterozygous for a *PTCH1* null mutation exhibit a high risk of cancers such as medulloblastomata, rhabdomyosarcomas and BCCs, confirming that *PTCH1* functions as a tumor suppressor. Currently, Ptch1$^{+/-}$ mice represent the most practical model of ultraviolet (UV)-mediated BCC formation. In addition to *PTCH1*, somatic mutations of *SMO* [19], a putative seven-transmembrane protein of the hedgehog pathway,

Figure 2. Components of the hedgehog pathway in mammals.



Three hedgehog homologs exist in humans: Shh, Ihh and Dhh. Hedgehog receptor PTCH1 (and its homolog PTCH2, not shown in this diagram) inhibits SMO signaling in the absence of hedgehog. Presence of hedgehog molecules, however, desuppresses SMO activities, which result in Gli-dependent transcription (three homologs exist in humans: Gli1, Gli2 and Gli3). Several other signaling molecules are also involved in hedgehog signaling, including Fu, Su(Fu), Rab23 and PKA. One unique feature of the hedgehog signaling pathway is the existence of several regulatory feedback loops: PTCH1, HIP and Gli1. However, signal transduction from SMO to Gli1 is still not very clear. Presently, the molecular link from hedgehog signaling activation to tumor formation is not known.

Dhh: Desert hedgehog; Fu: Fused kinase; HIP: hedgehog-interacting protein; Ihh: Indian hedgehog; PKA: Protein kinase A; PTCH: Patched; Shh: Sonic hedgehog; SMO: Smoothened; Su(Fu): Suppressor of fused.

occur in sporadic BCCs. Activation of the hedgehog pathway is also found in medulloblastomata, small-cell lung cancer and gastrointestinal tract cancers (Table 1) [20–24]. These findings provide additional insight into the role of the sonic hedgehog pathway in human cancer.

In summary, the hedgehog pathway is not only one of the most important signaling pathways during embryonic development, but is also highly activated in nearly 30% of all human cancers. Recent studies from several groups indicate an important role of the hedgehog pathway in prostate cancer [25–28]. The authors shall summarize recent findings regarding hedgehog signaling during prostate development and in prostate cancer.

Hedgehog signaling during formation of the prostate gland

Formation of the murine prostate gland is initiated through budding of the urogenital sinus epithelium (UGE) into the surrounding mesenchyme to form the main prostatic ducts in response to testosterone stimulation, which is followed by duct formation, elongation and branching. The mature prostate gland is an intensively branched ductal system enmeshed in a supporting stroma and connected to the urethra by approximately 25 main ducts. Expression of Shh and its target genes, *PTCH1* and *Gli1*, is observed in the prebudding stage; peaks in the budding stage and then declines during ductal branching [29–31]. In contrast, Shh is expressed in the UGE, Ptch1, Gli1, and Gli2 are localized primarily to mesenchyme surrounding prostate buds. These observations suggest that epithelial hedgehog expression activates stroma-mediated signals and stimulate epithelial cell proliferation in a paracrine manner.

Expression of *Gli1* and *Ptch1* can be induced by an exogenous Shh peptide in the intact urogenital sinus or in the isolated urogenital mesenchyme [29–31]. In the presence of Shh antibodies or the hedgehog signaling inhibitor cyclopamine, ductal morphogenesis is inhibited. *Shh* mutant fetuses display abnormal urogenital development and fail to form prostate buds. Thus, *Shh* is critical for maintaining appropriate prostate growth, proliferation and tissue polarity during development of the prostate gland.

It is suggested that prostate progenitor cells contain activated hedgehog signaling [27]. Prostate regeneration can be observed in rodent ventral prostate through castration-induced androgen withdrawal for 7 days, followed by androgen supplementation. The ventral prostate regenerates to its normal size after a 10-day course of androgen supplementation. However, inhibition of the hedgehog signaling by cyclopamine (or Shh antibodies) prevents prostate regeneration, yielding a prostate gland similar to the one after castration [27]. This study demonstrates that hedgehog signaling is critical for regeneration of adult prostate. Thus, hedgehog-positive prostate epithelial cells can be regarded as prostate progenitor cells.

Activation of hedgehog signaling in prostate cancer

Evidence from several groups indicates that the hedgehog signaling pathway is activated in subsets of prostate cancer, particularly metastatic tumors [25–28]. *In vivo* and *in vitro* data using cultured prostate cancer cell lines demonstrate

Table 1. Aberrant hedgehog signaling in human cancer.

Cancer	Gene mutations	Gene overexpression	Activation identified	Refs
Basal cell nevus syndrome	*PTCH1, Su(Fu)*		Yes	[16,17]
BCCs	*PTCH1, SMO*		Yes	[16,17,19]
Medulloblastomata	*PTCH1, Su(Fu), SMO*		Yes	[32]
Trichoepitheliomas	*PTCH1*		Yes	[35]
Gastric cancer		*Shh*	Yes	[22]
Esophageal cancer		*Shh*	Yes	[22]
Pancreatic cancer		*Shh*	Yes	[20,22]
Oral SCCs		*Shh*	Yes	[36]
Prostate cancer	*Su(Fu)*	*Shh*	Yes	[25–28]
SCLC		*Shh*	Yes	[21]

BCC: Basal cell carcinoma; PTCH: Patched; Shh: Sonic hedgehog; SCC: Small cell carcinoma; SCLC: Small cell lung carcinoma; SMO: Smoothened; Su(Fu): Suppressor of fused.

that inhibition of hedgehog signaling prevents cell growth, inhibits cell invasiveness and abrogates tumor metastasis in nude mice [25–28]. Thus, hedgehog signaling activation may be an important mechanism for the development and progression of prostate cancers.

Detection of hedgehog signaling activation in prostate cancers

Analyses of primary prostate cancers reveal elevated hedgehog signaling in subsets of prostate cancers [25–28]. Due to the heterogeneous nature of prostate cancer, a pure population of tumor cells is required for analysis of hedgehog target genes, which can be obtained through dissected transurethral resection of the prostate (TURP) or microdissected prostatectomy specimens. One group did not find a significant activation of the hedgehog target gene *Gli1* in the tumor samples [25], which could be due to a low percentage of tumor content in the specimens since only 5–30% of tumors exist in most prostatectomy specimens. Using real-time PCR analyses, elevated Gli1 and PTCH1 mRNA, indicators of the hedgehog signaling activation, were detected in all metastatic prostate tumors and subsets of locally metastasized tumors [25–28].

A large number of prostate cancer specimens can be readily analyzed by *in situ* hybridization and immunohistochemistry. *In situ* hybridization of Gli1 and PTCH1 indicates activation of the hedgehog pathway in subsets of prostate cancers. Expression of Gli1 mRNA is frequently detected in the epithelium [28], and stromal expression of Gli1 is also detected in some tumors [25]. Further thorough analyses using a large number of clinical specimens are needed to clarify these different observations. The authors suspect that these differences could be related to the known heterogeneity of prostate cancer. Immunohistochemistry studies indicate that expression of hedgehog target genes *PTCH1* and *HIP* is frequently observed in prostate cancers with high Gleason scores or distant metastases [26].

There are several mechanisms by which the hedgehog pathway can be activated. First, *Shh* expression is significantly higher in prostate cancers [25–28], suggesting that elevated *Shh* expression may contribute to the pathway activation in these tumors. However, *Shh* overexpression is unlikely to be the only trigger of hedgehog signaling activation since hedgehog signaling is tightly regulated by feedback mechanisms in a given cell (**Figure 2**).

Another possibility is the elevated expression of *SMO*, which is low in normal prostate [27]. The role of *SMO* for hedgehog signaling activation in prostate cancer cells is demonstrated in the prostate cell line Pre1 [27], which has no *SMO* expression and no hedgehog signaling activation. Following ectopic expression of *SMO*, hedgehog signaling activation is observed by luciferase reporter assays. This activity can be suppressed by cyclopamine or Shh antibodies. Further studies are needed to determine the mechanisms for *SMO* induction in primary prostate tumors.

The third mechanism is through mutations of other components of the hedgehog pathway. In the authors' studies, 11 of 27 PTCH1-positive prostate cancer specimens have no detectable Su(Fu) protein, a negative regulator of the hedgehog pathway [26]. Two of these 11 tumors were shown to contain *Su(Fu)* mutations [26]. Previously, mutations of *Su(Fu)* have been identified in a subset of medulloblastomata [32]. Thus, inactivation of the negative regulator *Su(Fu)* is another mechanism

for hedgehog signaling activation. The overall results from these studies indicate that the hedgehog pathway is frequently activated in advanced or metastatic prostate cancers.

Association of activated hedgehog signaling with cellular functions of prostate cancer

Several cell lines have been employed to demonstrate the involvement of hedgehog signaling for prostate cancer cellular functions, including PC3, LNCap, Du145, 22RV1 and several rodent prostate cancer cell lines. In comparison with immortalized prostate epithelial cell lines Pre1 and RWPE-1, all prostate cancer cell lines have relatively high levels of Gli1 and PTCH1 mRNA, suggesting increased hedgehog signaling. Thus, it is predicted that inhibition of the hedgehog pathway by the SMO antagonist, cyclopamine, would suppress cell proliferation and invasiveness.

Indeed, prostate cancer cells are often responsive to treatment of cyclopamine although variable inhibition is noted among different cell lines [25–28]. Following treatment with 5 μM cyclopamine in PC3 cells, expression of hedgehog target genes is dramatically suppressed, which is accompanied with a significant reduction in bromodeoxyuridine (BrdU)-positive cells. This effect is specific because addition of tomatidine, a nonspecific compound with a similar structure to cyclopamine, had no effect on either target gene expression or DNA synthesis [26]. On the other hand, the prostate epithelial cell line, RWPE-1, which demonstrates no activation of the hedgehog signaling pathway, was not sensitive to cyclopamine, indicating that cyclopamine specifically affects cells with elevated hedgehog signaling. As a result, cell growth of PC3 cells can be inhibited by cyclopamine, but not by tomatidine.

Prostate cancer progression is accompanied by increased cell invasiveness, and hedgehog signaling is frequently activated in advanced prostate cancers. It is therefore predicted that cyclopamine may inhibit cell invasiveness of prostate cancer cells. Cell invasiveness can be examined by the cell's ability to penetrate a matrix gel. In all prostate cancer cell lines, but not in the control RWPE-1 cells, the authors demonstrated that cyclopamine can reduce cell invasiveness by 70% [26]. Thus, hedgehog signaling activation regulates both cell proliferation and invasiveness of prostate cancer cells.

Evidence indicates that Gli1 plays an important role during hedgehog signaling activation in prostate cancers [25–28]. First, Gli1 is highly elevated in primary prostate tumors and cancer cell lines. Second, cancer cells with ectopic expression of *Gli1* are resistant to cyclopamine treatment [26,27]. Third, normal prostate epithelial cell lines Pre1 and AT2.1, are significantly more aggressive following ectopic expression of Gli1 [26,27]. Furthermore, inactivation of *Gli1* by short interfering RNA (siRNA) is sufficient to inhibit DNA synthesis in prostate cancer cells [28].

Hedgehog signaling & tumor growth in nude mice

In vitro data from several prostate cell lines predict the importance of the hedgehog pathway in tumor growth *in vivo*. Using LNCap cells, Fan and colleagues have demonstrated the dependence of *Shh* expression on tumor growth in nude mice [25]. Elegant experiments by Beachy and co-workers have provided direct evidence for Shh and tumor growth [27]. First, tumors derived from human prostate cancer cell lines PC3 and 22RV1 in nude mice are all sensitive to cyclopamine treatment. By day 28 following subcutaneous injection, mice develop tumors with a mean size of 155 mm^3. In contrast, mice treated with cyclopamine following cancer cell inoculation have no visible tumors. Ectopic expression of Gli1 renders these cells resistant to cyclopamine treatment in mice, supporting the specificity of the cyclopamine effects [27]. Second, these investigators demonstrate, using a series of rodent prostate cancer cell lines with different metastatic potentials, that hedgehog signaling activity is associated with tumor metastasis. The highly metastatic AT6.3 cells rapidly form tumors in nude mice. These tumors metastasize to the lung, resulting in death in a few weeks. Following intraperitoneal injection of cyclopamine, lung metastasis is dramatically decreased, and the mice survive for a longer period of time [27]. Conversely, AT2.1 cells, which have a low metastatic potential, do not metastasize to the lung following subcutaneous injection. However, ectopic expression of Gli1 in this cell line significantly increases metastasis, further supporting the role of hedgehog signaling in prostate cancer progression and metastasis [27].

Conclusion

Several recent studies demonstrate that the hedgehog pathway is activated in advanced prostate cancers, as indicated by high expression of hedgehog target genes. These results further demonstrate that hedgehog signaling is required for cell proliferation, invasion and tumor metastasis of prostate cancer cells. Thus, targeted inhibition of the hedgehog pathway should be effective in inhibiting prostate cancer progression. The first clinical trial using hedgehog signaling

inhibitor is already being initiated by Genentech and Curis, Inc. It is anticipated that, in the next few years, hedgehog signaling-based cancer clinical trials will be carried out by multiple pharmaceutical or biotechnology industries.

Future perspective

Although additional studies are needed to verify hedgehog signaling in a large number of prostate cancer specimens, recent studies provide a clear rationale for prostate cancer therapy using hedgehog signaling inhibitors.

Studies in other types of tumors have predicted the feasibility for cancer therapy using hedgehog signaling inhibitors. Using *p53*$^{-/-}$, *Ptch1*$^{+/-}$ mice as a model for medulloblastomata, hedgehog signaling inhibitors, HhAntag (Curis, Inc. and Genentech, Inc.) or cyclopamine, prevent tumor progression and prolong medulloblastoma-free survival of the mice [15,23,33]. Similarly, oral delivery of cyclopamine in drinking water prevents formation of UV-induced BCCs and reduces the number of mature BCCs [34]. In these experiments, long-term (20 weeks) treatment of mice with cyclopamine had no effect on the overall survival, suggesting that cyclopamine has few side effects. A pilot study using cyclopamine in patients with BCCs has also proven to be beneficial in the treatment of these cancers [35].

Currently, our understanding of the roles of the hedgehog pathway in prostate cancer is rather limited. Thus, several important issues need to be addressed before clinical therapeutics can be effectively performed. First, we need to:

- Understand how the hedgehog pathway interacts with other signaling pathways in prostate cancers
- Demonstrate in a model system that activation of the hedgehog pathway is sufficient to drive cancer progression in an organism [6,9]
- Understand the mechanism(s) by which inhibition of hedgehog signaling prevent tumor progression

If encouraging results are obtained in these studies and following approval by the US Food and Drugs Administration (FDA), it will be possible to test the effectiveness of these inhibitors in clinical trials.

There are several considerations in the future treatment of prostate cancers using hedgehog inhibitors. First, hedgehog signaling should be tested in the needle-core biopsies, which can be best utilized for *in situ* hybridization. Another possibility is to establish an enzyme-linked immunosorbent based assay using patient's serum. Future studies in identifying sensitive and cost-effective methods for detecting hedgehog signaling will be extremely helpful.

Second, an effective agent should be selected. Currently, there are several possibilities: Shh antibodies, cyclopamine or its analogs, and Gli1 siRNA [25–27]. Careful examination of Shh in normal prostate epithelium should be assessed before applying the antibodies because Shh is expressed in both the tumor and some normal epithelium of the prostate [25]. Although cyclopamine or its analog KAAD-cyclopamine is effective in suppressing hedgehog signaling activation, a high concentration (at a micromolar concentration range) is often needed to observe biologic effects in cultured cells. Therefore, novel inhibitors with efficacy in the nanomolar ranges are needed for clinical application. Since cyclopamine mainly targets SMO, tumors with alterations downstream of SMO are expected to be resistant to cyclopamine. In fact, the authors have demonstrated inactivation of *Su(Fu)* in prostate cancers and other tumors [Unpublished Data] [26]. One possibility is to use Gli1 siRNA, since recent data indicate that downregulation of Gli1 may be an important mechanism by which inhibition of the hedgehog pathway by cyclopamine induces apoptosis [26,27]. Sanchez and colleagues also indicated that Gli1 siRNA downregulated BrdU incorporation in prostate cancer cells. Future improvements of current *in vivo* delivery systems will be necessary for siRNA-based therapeutics [28].

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Jemal A, Murray T, Ward E *et al.*: Cancer statistics, 2005. *CA Cancer J. Clin.* 55(1), 10–30 (2005).
2. Deutsch E, Maggiorella L, Eschwege P *et al.*: Environmental, genetic, and molecular features of prostate cancer. *Lancet Oncol.* 5(5), 303–313 (2004).
3. Humphrey PA: Gleason grading and prognostic factors in carcinoma of the prostate. *Mod. Pathol.* 17(3), 292–306 (2004).
4. Dong JT: Chromosomal deletions and tumor suppressor genes in prostate cancer. *Cancer Met. Rev.* 20(3–4), 173–193 (2001).
5. Gonzalgo ML, Isaacs WB: Molecular pathways to prostate cancer. *J. Urol.* 170(6 Pt 1), 2444–2452 (2003).
6. Wang S, Gao J, Lei Q *et al.*: Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer. *Cancer Cell* 4(3), 209–221 (2003).
   • **The tumor suppressor role of *PTEN* in prostate cancer is demonstrated in a murine model.**
7. Henrique R, Jeronimo C: Molecular detection of prostate cancer: a role for GSTP1 hypermethylation. *Eur. Urol.* 46(5), 660–669. Discussion 669 (2004).

Executive summary

Prostate cancer

- Prostate cancer is the most common malignancy and the second leading cancer-related cause of death in men in the USA. Despite enormous efforts in our understanding of the molecular basis of prostate cancer, very little progress has been made in prevention and treatment of this often lethal cancer. Recent studies have demonstrated that hedgehog signaling is frequently activated in advanced or metastatic prostate cancers.

Hedgehog signaling in human cancers

- The hedgehog pathway, initially identified in the fruit fly, is a master regulator of cell proliferation, tissue differentiation and polarity. The link between the hedgehog pathway and human cancer was first identified in the Gorlin syndrome, a disease with a high risk of basal cell carcinomas (BCCs) and medulloblastomata.
- Loss-of-function mutations of the tumor suppressor patched (*PTCH1*) gene or gain-of-functions of proto-oncogene smoothened (*SMO*) are the causes of hedgehog signaling activation in BCCs and medulloblastomata.
- Activation of the hedgehog pathway is also found in small-cell lung cancer, pancreatic cancer and gastrointestinal tract cancers.
- Recent data from several groups indicate that hedgehog signaling is activated in advanced prostate cancers.

Hedgehog signaling during formation of the prostate gland

- During development of the prostate gland, sonic hedgehog (*Shh*) is important for maintaining appropriate prostate growth, proliferation and tissue polarity. Evidence also suggests that hedgehog signaling is critical for regeneration of adult prostate.

Activation of hedgehog signaling in prostate cancer

- Analyses of primary prostate cancers reveal elevated hedgehog signaling in subsets of prostate cancers, particularly advanced and metastatic prostate cancers.
- There are several mechanisms by which the hedgehog pathway can be activated: overexpression of *Shh*, elevated expression of *SMO* or downregulation of *Su(Fu)*. Using a specific hedgehog signaling inhibitor cyclopamine, in cultured cell lines, it has been demonstrated that hedgehog signaling activation is required for cell proliferation and cancer cell invasiveness.
- Published *in vitro* data demonstrates that tumor formation and metastases of prostate cancer cells in nude mice are dependent on hedgehog signaling. Conversely, ectopic expression of Gli1, the downstream effector of the hedgehog pathway, in a nontumorigenic prostate epithelial cell line is sufficient to cause tumor formation in nude mice. Experiments using specific short interfering RNA of Gli1 demonstrate the importance of hedgehog signaling for DNA synthesis in prostate cancer cells.
- These results demonstrate that hedgehog signaling is required for cell proliferation, invasion and tumor metastasis of prostate cancer cells. Thus, targeted inhibition of the hedgehog pathway should be effective in inhibiting prostate cancer progression.

8. De Marzo AM, Meeker AK, Zha S *et al.*: Human prostate cancer precursors and pathobiology. *Urology* 62(5 Suppl. 1), 55–62 (2003).
9. Gao H, Ouyang X, Banach-Petrosky W *et al.*: A critical role for $p27^{kip1}$ gene dosage in a mouse model of prostate carcinogenesis. *Proc. Natl Acad. Sci. USA* 101(49), 17204–17209 (2004).
   - **The synergism between *PTEN* and *p27* during prostate cancer metastasis is demonstrated in a murine model.**
10. Taipale J, Beachy PA: The hedgehog and Wnt signaling pathways in cancer. *Nature* 411(6835), 349–354 (2001).
11. Chuang PT, McMahon AP: Vertebrate hedgehog signaling modulated by induction of a hedgehog-binding protein. *Nature* 397(6720), 617–621 (1999).
12. Pasca di Magliano M, Hebrok M: Hedgehog signaling in cancer formation and maintenance. *Nature Rev. Cancer* 3(12), 903–911 (2003).
13. Bale AE: Sheep, lilies and human genetics. *Nature* 406(6799), 944–945 (2000).
14. Frank-Kamenetsky M, Zhang XM, Bottega S *et al.*: Small-molecule modulators of Hedgehog signaling: identification and characterization of Smoothened agonists and antagonists. *J. Biol.* 1(2), 10 (2002).
15. Romer JT, Kimura H, Magdaleno S *et al.*: Suppression of the Shh pathway using a small molecule inhibitor eliminates medulloblastoma in $Ptc1^{(+/-)}p53^{(-/-)}$ mice. *Cancer Cell* 6(3), 229–240 (2004).
    - **Demonstrates that targeted inhibition of hedgehog signaling is sufficient to prevent sporadic medulloblastomas in a murine model.**
16. Hahn H, Wicking C, Zaphiropoulous PG *et al.*: Mutations of the human homolog of Drosophila patched in the nevoid basal cell carcinoma syndrome. *Cell* 85(6), 841–851 (1996).
17. Johnson RL, Rothman AL, Xie J *et al.* Human homolog of patched, a candidate gene for the basal cell nevus syndrome. *Science* 272(5268), 1668–1671 (1996).
18. Dahmane N, Lee J, Robins P, Heller P, Ruiz i Altaba A: Activation of the transcription factor Gli1 and the Sonic hedgehog signaling pathway in skin tumours. *Nature* 389(6653), 876–881 (1997).
19. Xie J, Murone M, Luoh SM *et al.*: Activating Smoothened mutations in sporadic basal-cell carcinoma. *Nature* 391(6662), 90–92 (1998).
20. Thayer SP, Di Magliano MP, Heiser PW *et al.*: Hedgehog is an early and late mediator of pancreatic cancer tumorigenesis. *Nature* 425(6960), 851–856 (2003).
    - **Demonstrates that hedgehog signaling is involved in pancreatic cancers.**
21. Watkins DN, Berman DM, Burkholder SG *et al.*: Hedgehog signaling within airway epithelial progenitors and in small-cell lung cancer. *Nature* 422(6929), 313–317 (2003).
    - **A role of hedgehog signaling in human small-cell lung cancer is revealed in this study.**
22. Berman DM, Karhadkar SS, Maitra A *et al.*: Widespread requirement for Hedgehog ligand stimulation in growth of digestive tract tumours. *Nature* 425, 846–851 (2003).
    - **The role of hedgehog signaling activation is shown to be involved in gastrointestinal tract cancers.**

▶23. Berman DM, Karhadkar SS, Hallahan AR *et al.*: Medulloblastoma growth inhibition by hedgehog pathway blockade. *Science* 297(5586), 1559–1561 (2002).

• **Reports that inhibition of hedgehog signaling by cyclopamine causes regression of medulloblastomas in nude mice.**

▶24. Dahmane N, Sanchez P, Gitton Y *et al.*: The sonic hedgehog-Gli pathway regulates dorsal brain growth and tumorigenesis. *Development* 128(24), 5201–5212 (2001).

• **First report of cyclopamine effects on medulloblastoma cells.**

▶25. Fan L, Pepicelli CV, Dibble C *et al.*: Hedgehog signaling promotes prostate xenograft tumor growth. *Endocrinology* 145, 3961–3970 (2004).

• **First report showing that prostate xenograft tumor growth is dependent on hedgehog signaling.**

▶26. Sheng T, Li C, Zhang X *et al.*: Activation of the hedgehog pathway in advanced prostate cancer. *Mol. Cancer* 3(1), 29 (2004).

•• **Demonstrates that hedgehog signaling is activated in advanced prostate cancers. This report reveals that overexpression of Shh or lack of Su(Fu) expression occurs in tumors with activated hedgehog signaling. Inactivated mutations of *Su(Fu)* are revealed in two tumors. The relevance of hedgehog signaling to DNA synthesis and cell invasiveness is demonstrated in several cancer cell lines.**

▶27. Karhadkar SS, Bova GS, Abdallah N *et al.*: Hedgehog signaling in prostate regeneration, neoplasia and metastasis. *Nature* 431(7009), 707–712 (2004).

•• **Comprehensive study demonstrating that hedgehog signaling is activated in metastatic prostate cancers. In addition to overexpression of Shh, elevated *SMO* expression is shown in prostate cancer cells. The relevance of hedgehog signaling to DNA synthesis, cell invasiveness and tumor growth of prostate cancer cells is demonstrated in several cancer cell lines. In addition, evidence is provided to suggest that Shh positive cells may represent prostate stem cells.**

▶28. Sanchez P, Hernandez AM, Stecca B *et al.*: Inhibition of prostate cancer proliferation by interference with Sonic hedgehog-GLI1 signaling. *Proc. Natl Acad. Sci. USA* 101, 12561–12566 (2004).

•• **Demonstrates that hedgehog signaling is activated in subsets of prostate cancers. Overexpression of *Shh* is shown in tumors with activated hedgehog signaling. The relevance of hedgehog signaling to DNA synthesis of prostate cancer cells is shown in several cancer cell lines.**

▶29. Lamm ML, Catbagan WS, Laciak RJ *et al.*: Sonic hedgehog activates mesenchymal Gli1 expression during prostate ductal bud formation. *Dev. Biol.* 249(2), 349–366 (2002).

▶30. Freestone S, Marker P, Grace OC *et al.*: Sonic hedgehog regulates prostatic growth and epithelial differentiation. *Dev. Biol.* 264, 352–362 (2003).

▶31. Pu Y, Huang L, Prins GS: Sonic hedgehog-patched Gli signaling in the developing rat prostate gland: lobe-specific suppression by neonatal estrogens reduces ductal growth and branching. *Dev. Biol.* 273(2), 257–275 (2004).

▶32. Taylor MD, Liu L, Raffel C *et al.*: Mutations in SUFU predispose to medulloblastoma. *Nature Genet.* 31(3), 306–310 (2002).

▶33. Sanchez P, Ruiz IAA: *In vivo* inhibition of endogenous brain tumors through systemic interference of Hedgehog signaling in mice. *Mech. Dev.* 122(2), 223–230 (2005).

•• **The effectiveness of cyclopamine in preventing medulloblastoma in a murine model is reported in this paper.**

▶34. Athar M, Li C, Tang X *et al.*: Inhibition of smoothened signaling prevents ultraviolet B-induced basal cell carcinomas through regulation of Fas expression and apoptosis. *Cancer Res.* 64(20), 7545–7552 (2004).

•• **Demonstrates that long-term chronic treatment of mice with BCCs with cyclopamine prevents formation of microscopic tumors and regresses macroscopic tumors. Despite long-term treatment, no specific side effects (hair loss or changes of overall animal survival) are identified, suggesting a feasibility of cyclopamine for treatment of human cancers. Furthermore, a mechanism of cyclopamine-induced apoptosis in BCCs is reported.**

▶35. Tabs S, Avci O: Induction of the differentiation and apoptosis of tumor cells *in vivo* with efficiency and selectivity. *Eur. J. Dermatol.* 14(2), 96–102 (2004).

•• **First report of a pilot human clinical trial with cyclopamine on human BCCs. The result indicates that cyclopamine treatment is beneficial to basal cell carcinoma patients.**

▶36. Vorechovsky I, Unden AB, Sandstedt B, Toftgard R, Stahle-Backdahl M: Trichoepitheliomas contain somatic mutations in the overexpressed PTCH gene: support for a gatekeeper mechanism in skin tumorigenesis. *Cancer Res.* 57, 4677–4681 (1997).

▶37. Koike C, Mizutani T, Ito T *et al.*: Introduction of wild-type patched gene suppresses the oncogenic potential of human squamous cell carcinoma cell lines including A431. *Oncogene* 21, 2670–2678 (2002).

Affiliation

• *Jingwu Xie*
University of Texas Medical Branch at Galveston, Sealy Centers for Cancer Cell Biology and Environmental Health, Department of Pharmacology and Toxicology,
301 University Blvd, Galveston, TX 77555-1048, USA
Tel.: +1 409 747 1935;
Fax: +1 409 747 1938;
jinxie@utmb.edu